

## ADDENDUM

### Abbreviations to be added to page xvii:

[ <sup>3</sup> H]-CTOP	[ <sup>3</sup> H]-H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH <sub>2</sub>
[ <sup>3</sup> H]-DADL	[ <sup>3</sup> H]-DADLE
[ <sup>3</sup> H]-DADLE	[ <sup>3</sup> H]-D-Ala <sup>2</sup> -D-Leu <sup>5</sup> -Enkephalin
[ <sup>3</sup> H]-DAGO	[ <sup>3</sup> H]-Tyr-D-Ala-Gly-MePhe-Gly-ol
[ <sup>3</sup> H]-DAMGO	[ <sup>3</sup> H]-DAGO
[ <sup>3</sup> H]-DPDPE	[ <sup>3</sup> H]-[D-Pen <sup>2</sup> ,D-Pen <sup>5</sup> ]-Enkephalin
[ <sup>3</sup> H]-DSLET	[ <sup>3</sup> H]-D-Ser <sup>2</sup> -Leu <sup>3</sup> -Thr <sup>6</sup> -Enkephalin
[ <sup>3</sup> H]-EKC	[ <sup>3</sup> H]-ethylketocyclazocine
[ <sup>125</sup> I]-FK 33-824	[ <sup>125</sup> I]-D-[Ala <sup>2</sup> -Me-Phe <sup>4</sup> -met(o)-ol]-Enkephalin
[ <sup>3</sup> H]-U69-593	[ <sup>3</sup> H]-((5a,7a,8b)-(+)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)-benzeneacetamide)

### Chapter 3

page 74, paragraph 3, line 5/6:

“and RVLM (+28%)” *should read* “and decreased in the RVLM (-28%)”

page 89, paragraphs 2 and 3:

*delete paragraphs 2 and 3 and replace with:*

“The RVLM is the third relay nucleus in the central sympathetic loop that has a pivotal role in the control of cardiovascular homeostasis (Dampney, 1994). In the present study, prepro-ENK mRNA was found to be significantly decreased in the RVLM of SHR compared to WKY. These results are in agreement with the findings of Boone and colleagues (Boone & McMillen, 1994b), where SHR were found to have a reduced expression of prepro-ENK mRNA in the RVLM, CVLM and NTS when compared to WKY. Regions implicated in central cardiovascular control that receive enkephalin-containing projections from the RVLM, such as the LC and spinal cord, may also be affected by an altered expression of prepro-ENK mRNA in RVLM neurons (Menetrey & Basbaum, 1987; Drolet *et al.*, 1992; Guyenet *et al.*, 2001). Thus, decreased expression of prepro-ENK mRNA in the RVLM may contribute to the elevated BP status of SHR when compared to their normotensive WKY controls.”

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..... *Carol M Williams* .....

*for* Sec. Research Graduate School Committee

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**ACUTE AND CHRONIC RESTRAINT:  
IMPACT ON CENTRAL NEUROPEPTIDE  
SYSTEMS**

A thesis submitted to the Faculty of Medicine  
Monash University  
for the Degree of Doctor of Philosophy

By

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April, 2001

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## Voor degene waar ik van houd

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**Life affords no higher pleasure than that of surmounting difficulties, passing from one step of success to another, forming new wishes and seeing them gratified.**

Samuel Johnson (1709-1784)  
English lexicographer, critic and writer

# Stress stres

Noun - 1. the sum of the biological reactions to any adverse stimulus, physical, mental, or emotional, internal or external, that tends to disturb the organism's homeostasis.  
2. the stimuli that elicit stress reactions.



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## SUMMARY

The present thesis investigated the neurochemical changes in the opioid, galanin (GAL) and neuropeptide Y (NPY) systems in the rat central nervous system (CNS) induced by exposure to a restraint stress paradigm. More specifically, restraint-induced changes in prepro-enkephalin (prepro-ENK), proDynorphin (proDYN), prepro-galanin (prepro-GAL) and prepro-neuropeptide Y (prepro-NPY) mRNA expression, and in the density of opioid ( $\mu$ ,  $\delta$  and  $\kappa$ ) and GAL receptors, were quantified and compared in selected regions of the CNS of normotensive Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats. In addition, the role of the opioid system in the neural response to a single period of restraint was investigated in WKY to determine whether the changes in gene expression and receptor density previously observed in the thesis contribute to the central stress response.

Using oligonucleotide probes and a standard *in situ* hybridisation histochemistry protocol, chapter 3 investigated the central expression of prepro-ENK, proDYN, prepro-GAL and prepro-NPY mRNA in WKY and SHR in the resting and stressed state. Significant neuropeptide- and region-specific changes in expression were observed for each of these neuropeptide precursors in multiple brain regions of SHR when compared to WKY. Examples of discrete CNS regions exhibiting significant alterations in gene expression in SHR compared to WKY include the supraoptic nucleus (SON; proDYN, prepro-GAL), arcuate nucleus (ARC; prepro-NPY), dentate gyrus (DG; proDYN), rostral ventrolateral medulla (RVLM; prepro-ENK, prepro-GAL) and nucleus of the solitary tract (NTS; proDYN).

WKY and SHR rats were exposed to a 60 min session of restraint for 0, 1, 3, 5 or 10 consecutive days and the subsequent effects on gene expression were determined in chapter 3. In WKY, 1 session of restraint induced significant changes in expression of neuropeptide precursor mRNA in regions such as the central nucleus of the amygdala (Ce; prepro-ENK, prepro-GAL), ventromedial hypothalamic nucleus (VMH; proDYN), perifornical nucleus of the hypothalamus (PeF; prepro-ENK) and ARC (prepro-NPY), while regions such as the VMH (proDYN), locus coeruleus (LC; prepro-ENK), RVLM (prepro-ENK) and NTS (prepro-ENK) in WKY exhibited a significantly altered level of gene expression after periods of repeated restraint. In contrast in SHR, acute restraint produced significant changes in the SON (proDYN),

Ce (proDYN), LC (prepro-GAL) and VLM (prepro-NPY). A number of regions in the CNS of SHR were sensitive to chronic restraint, including the SON (proDYN, prepro-GAL), Ce (proDYN, prepro-GAL), LC (prepro-ENK, prepro-NPY) and NTS (proDYN). Following comparison of the temporal response profile between WKY and SHR, significant differences between strains were observed in the PeF (prepro-ENK), SON (proDYN, prepro-GAL), ARC (prepro-NPY), Ce (proDYN, prepro-GAL) and LC (prepro-ENK).

In chapter 4, *in vitro* receptor autoradiography was used to visualise  $\mu$ -opioid ( $[^{125}\text{I}]\text{-FK 33-824}$ ),  $\delta$ -opioid ( $[^3\text{H}]\text{-naltrindole}$ ),  $\kappa$ -opioid ( $[^3\text{H}]\text{-U69-593}$ ) and GAL ( $[^{125}\text{I}]\text{-GAL}$ ) receptors in the CNS of WKY and SHR. The basal density of each of these receptors were compared between strains, and significant differences were detected in regions such as the paraventricular nucleus of the hypothalamus (PVN;  $\kappa$ ), thalamus ( $\mu$ ), basolateral nucleus of the amygdala (BL;  $\mu$ ), medial nucleus of the amygdala (Me;  $\kappa$ ), caudal ventrolateral medulla (CVLM;  $\delta$ ) and NTS ( $\delta$ ,  $\kappa$ ). Thus, the results of chapters 3 and 4 demonstrate that gene expression and receptor density in the opioid, GAL and NTS systems are significantly different between WKY and SHR. Moreover, as some of the significant differences between strains are localised in brain regions reported to be implicated in the control of blood pressure (BP), nociception and movement, they may contribute to the phenotypic variations previously observed in SHR.

Similar to chapter 3, the experiments outlined in chapter 4 compared the density of receptors for GAL and the 3 opioid receptor subtypes in the CNS of WKY and SHR rats exposed to a 10 day restraint paradigm that consisted of a single 60 min restraint session per day. In WKY, acute restraint induced significant changes in receptor density in regions such as the VMH ( $\delta$ ), Ce ( $[^{125}\text{I}]\text{-GAL}$ ), Me ( $[^{125}\text{I}]\text{-GAL}$ ), basomedial nucleus of the amygdala (BM;  $\delta$ ) and cortex ( $\mu$ ). Furthermore, significant alterations in receptor density were observed after repeated exposure to restraint in WKY in regions such as the VMH ( $\delta$ ,  $[^{125}\text{I}]\text{-GAL}$ ), Ce ( $[^{125}\text{I}]\text{-GAL}$ ), parabrachial nucleus (PB;  $\mu$ ) and medial NTS ( $\kappa$ ). Different regions in SHR exhibited significant changes to both acute and chronic restraint stress. Following acute restraint, the PVN ( $\kappa$ ), Me ( $\kappa$ ), BL ( $\mu$ ) and NTS ( $\delta$ ) of SHR contained significantly altered receptor density, while after periods of repeated restraint, significant changes in receptor density were detected in the PVN ( $\kappa$ ), Me ( $\kappa$ ), BL ( $\mu$ ), lateral PB ( $[^{125}\text{I}]\text{-GAL}$ ), NTS ( $\delta$ ,  $\kappa$ ) and CVLM ( $\delta$ ) when compared to unstressed SHR. Statistical comparison of the temporal response to the restraint stress paradigm between WKY and SHR revealed that regions such as the BL ( $\mu$ ), Ce ( $[^{125}\text{I}]\text{-GAL}$ ), PB ( $\mu$ ), LC ( $\delta$ ) and NTS ( $\delta$ ) exhibited a significantly different response profile between strains.

The results described above clearly show that psychological stressors such as restraint can induce significant changes in gene expression or receptor density in the central opioid, GAL and NPY systems, and furthermore, these changes are dependent on the rat strain, neuropeptide and region under investigation, as well as the duration of exposure to the stressor. These data also demonstrate that the neural response to restraint is significantly altered in the CNS of SHR when compared to WKY, and the findings may provide a basis for further experiments that correlate restraint-induced changes in CNS regions that modulate particular physiological functions (such as those described in chapters 3 and 4) with the atypical physiological response to stress previously observed in SHR.

As described above, restraint produced significant alterations in the expression of opioid peptide precursors, as well as the density of the 3 opioid receptor subtypes in the CNS of WKY. Chapter 5 investigated whether the significant changes in the opioid system induced by acute exposure to restraint were translated into changes in neuronal activity in the same nucleus or in the regions that receive efferent projections from that particular nucleus. Thus, the non-selective opioid receptor antagonist, naloxone, was injected centrally (i.c.v.; 100nmol in 5 $\mu$ l) to block all opioid receptor subtypes in the CNS of rats that were subsequently exposed to a single 60 min session of restraint. One hour after conclusion of the restraint paradigm, the rats were anaesthetised (sodium pentobarbitone; 80mg/kg; i.p.), transcardially perfused, brains processed for Fos immunohistochemistry using a standard protocol and the numbers of Fos-positive cells in selected regions were quantified. Fos, an immediate early gene, is a commonly used marker of neuronal activation.

In unstressed WKY rats, 100nmol naloxone (i.c.v.) had no significant effect on resting MAP or on Fos expression in regions such as the PVN, Ce, Me, RVLM and NTS in unstressed rats. In rats exposed to a single period of restraint, i.c.v. naloxone significantly increased the number of Fos-positive cells in the parvocellular subregion of the PVN (pPVN), while the number of Fos-positive cells in the magnocellular SON were significantly reduced in the SON of naloxone-pretreated rats when compared to restrained rats receiving i.c.v. saline. In regions such as the Me, Ce, LC, RVLM and NTS, naloxone had no significant effect on Fos production during exposure to restraint. These results therefore suggest that the restraint-induced release of opioids within the CNS is apparently producing a net inhibition of neurons in the pPVN and a net activation of SON neurons. Thus, considering that the pPVN and SON have been implicated in the modulation of the release of hormones such as vasopressin, oxytocin and corticotrophin-releasing factor, as well as the modulation of sympathetic outflow, the opioid system may be

modulating the function of any one of these systems during restraint. Further experiments investigating the functional effects of antagonism of opioid receptors in the pPVN and SON during restraint are therefore required to gain a greater understanding of the role of the opioid system in the response to restraint and other stressors.

Furthermore, the restraint-induced changes in the PeF (prepro-ENK) and VMH (proDYN) of WKY observed in chapter 3 may correlate with the results of chapter 5, as both of these regions have been shown to project to the pPVN. These data therefore demonstrate that at least some of the neurochemical alterations observed in the CNS of rats exposed to acute restraint may be contributing to one or more components of the stress response. Further experiments that focus on the GAL and NPY system will determine the level of their involvement in the various facets of the response to stressors such as restraint.

In conclusion, the present thesis clearly demonstrates that acute and chronic restraint stress can induce dynamic, significant changes in the activity of a number of central neuropeptide systems in both WKY and SHR, and these changes may contribute to the modulation of a variety of neural and physiological components of the stress response.

# ABBREVIATIONS

## 1.1 GENERAL ABBREVIATIONS

3V	Third ventricle
AcAnh	Acetic Anhydride
ACh	Acetylcholine
ACTH	Adrenocorticotrophic hormone
AM/Str	Amygdalostratial transition zone
ANOVA	Analysis of Variance
AP	Area postrema
A-P	Anterior-Posterior
ARC	Arcuate nucleus
AV	Anteroventral thalamic nucleus
BHR	Borderline hypertensive rat
BL	Basolateral nucleus of the amygdala
BM	Basomedial nucleus of the amygdala
B <sub>MAX</sub>	Total number of binding sites
BNST	Bed nucleus of the stria terminalis
BP	Blood pressure
bp	Base pairs
bpm	Beats per minute
BSA	Bovine Serum Albumin
Ca <sup>2+</sup>	Calcium ions
cAMP	Cyclic 3',5'-adenosine monophosphate
Cb	Cerebellum
cc	Central canal
cDNA	Complementary deoxyribonucleic acid
Ce	Central nucleus of the amygdala
CHO	Chinese Hamster Ovary (cells)
Cing	Cingulate cortex
CL	Centrolateral nucleus of the thalamus

CLIP	Corticotrophin-like intermediate lobe peptide
CM	Centromedial nucleus of the thalamus
CnF	Cuneiform nucleus
CNS	Central nervous system
Co	Cortical amygdaloid nucleus
comm NTS	Commissural nucleus of the solitary tract
CPON	C-terminal flanking peptide of neuropeptide Y
CPu	Caudate putamen (striatum)
CRF	Corticotrophin-releasing factor/hormone
CTX	Cortex
Cu	Cuneate nucleus
cu	Cuneate fasciculus
CVLM	Caudal ventrolateral medulla
DAB	3',3'-diaminobenzidine
dATP	Deoxyadenosine 5'-triphosphate
DEn	Dorsal endopiriform nucleus
DEPC	Diethyl pyrocarbonate
DG	Dentate gyrus
dIPAG	Dorsolateral periaqueductal gray
DM	Dorsomedial hypothalamic nucleus
DMX	Dorsal motor nucleus of the vagus
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
DPM	Disintegrations per minute
DR	Dorsal raphe
DRet	Dorsal reticular thalamus
DRG	Dorsal root ganglia/ganglion
D-V	Dorsal-Ventral
DVC	Dorsal vagal complex
ECu	External cuneate nucleus
Ent	Entorhinal cortex
EP	Entopeduncular nucleus
EPSP	Excitatory postsynaptic potential
EtOH	Ethanol
Fr	Frontal cortex

GABA	$\gamma$ -Aminobutyric acid
GAD	Glutamic acid decarboxylase
GAL	Galanin
GIRK	G protein-coupled inwardly rectifying K <sup>+</sup> channels
GMAP	Galanin message-associated peptide
Gr	Gracile nucleus
Hab	Habenula
Hipp	Hippocampus
HPA axis	Hypothalamo-pituitary-adrenal axis
HR	Heart rate
i.a.	Intra-arterial
IC	Intercalated nuclei of the amygdala
i.c.v.	Intracerebroventricular
IEG	Immediate early gene
IML	Intermediolateral cell column
IOC	Inferior olivary complex
i.p.	Intraperitoneal
i.v.	Intravenous
ir	Immunoreactivity
IRt	Intermediate reticular nucleus
ISHH	<i>In situ</i> hybridisation histochemistry
K <sup>+</sup>	Potassium ions
kb	Kilobases
K <sub>d</sub>	Dissociation constant
Lat PB	Lateral parabrachial nucleus
LC	Locus coeruleus
LD	Laterodorsal nucleus of the thalamus
LDTg	Laterodorsal tegmental nucleus
LHy	Lateral hypothalamus
LRtPC	Lateral reticular nucleus; parvocellular
LSO	Lateral superior olive
LV	Lateral ventricle
MAP	Mean arterial pressure
MCID	Micro Computing Imaging Device
MDL	Mediodorsal thalamic nucleus, lateral

Me	Medial nucleus of the amygdala
med NTS	Medial nucleus of the solitary tract
med PB	Medial parabrachial nucleus
min	Minutes
ml	Medial lemniscus
M-L	Medial-Lateral
Mo5	Motor trigeminal nucleus
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone
MTu	Medial tuberal nucleus
NAcc	Nucleus accumbens
NAdr	Noradrenaline
NAmb	Nucleus ambiguus
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NRS	Normal Rabbit Serum
NTS	Nucleus of the solitary tract
OB	Olfactory bulb
PAG	Periaqueductal (central) gray
Par	Parietal cortex
PB	Parabrachial nucleus
PBS	Phosphate-buffered saline
pd	Predorsal bundle
Pe	Periventricular nucleus of the hypothalamus
PeF	Perifornical nucleus of the hypothalamus
PFA	Paraformaldehyde
Pir	Piriform cortex
PNMT	Phenylethanolamine <i>N</i> -methyl transferase
PnC	Pontine reticular nucleus, caudal
PnV	Pontine reticular nucleus, ventral
POA	Preoptic area
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
pPVN	Parvocellular paraventricular nucleus of the hypothalamus
Prepro-ENK	Prepro-enkephalin

Prepro-GAL	Prepro-galanin
Prepro-NPY	Prepro-neuropeptide Y
PrH	Prepositus hypoglossi
ProDYN	Prodynorphin
PVA	Paraventricular nucleus of the thalamus
PVN	Paraventricular nucleus of the hypothalamus
PYY	Peptide YY
Re	Reuniens nucleus
Ret	Reticular thalamic nucleus
Rh	Rhomboid thalamic nucleus
RMag	Raphe magnus
RNA	Ribonucleic acid
RPa	Raphe pallidus
rpm	Revolutions per minute
RS	Retrosplenic cortex
Rt	Reticular field
RVLM	Rostral ventrolateral medulla
RVMM	Rostral ventromedial medulla
s.c.	Subcutaneous
SD	Sprague Dawley rat
S.E.M.	Standard error of the mean
SHR	Spontaneously Hypertensive rat
SON	Supraoptic nucleus
SOR	Supraoptic nucleus, retrochiasmatic
sp5	Spinal trigeminal tract
Sp5C	Spinal trigeminal nucleus; caudal
SSC	Saline sodium citrate
st	Stria terminalis
Strep-HRP	Streptavidin-horseradish peroxidase
SubC	Subcoeruleus
TEA	Triethanolamine
Teg	Tegmental area
TH	Tyrosine hydroxylase
Thal	Thalamus
Tz	Nucleus of the trapezoid body

tz	Trapezoid body
VIFP	Victorian Institute of Forensic Pathology
VLM	Ventrolateral medulla
vIPAG	Ventrolateral periaqueductal gray
VMH	Ventromedial hypothalamic nucleus
VP	Ventral posterior thalamic nucleus
VTA	Ventral tegmental area
WKY	Wistar Kyoto rat
XII	Hypoglossal nucleus
ZI	Zona incerta

## 1.2 AMINO ACIDS

AMINO ACID	SYMBOL		AMINO ACID	SYMBOL	
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic Acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamic Acid	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

## 1.3 NUCLEOTIDE BASES

ADENINE	A	GUANINE	G
CYTOSINE	C	THYMINE	T

## DECLARATION

The material contained herein has not been presented for the award of any other degree or diploma in any University or other institution. The research conducted has been carried out solely by the candidate, and this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I certify that the writing of this thesis, the results, interpretations, opinions and suggestions presented are my own work.



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support and belief in me. My friends also deserve a thankyou, as my poor attendance at BBQs, indoor soccer, golf and work over the past year leaves a lot to be desired. Thanks for your understanding, and now that the thesis has been lifted off my shoulders, I can't wait to take full advantage of the huge amount of spare time I now possess. Go Bombers.

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This acknowledgments section is one of the final parts of the thesis that remains, and as I write this paragraph, I have a feeling that may be akin to the sporting dream of many people: walking down the 18<sup>th</sup> fairway of Augusta towards the final green after hitting my 6 iron approach shot stone dead, leaving myself a 3 foot putt to claim the US Masters championship. It is even more difficult to put into words the enjoyment I have had during the past 4 years in the Department of Pharmacology. But, as a good friend of mine called Jake would say –

**IT'S ALL GOOD**

# GENERAL INTRODUCTION

.....

## Restraint Re-strānt'

Noun - The act of restraining, or holding back, controlling or checking. The state or fact of being restrained; confinement.

# CHAPTER 1

## GENERAL INTRODUCTION

### 1.1 STRESS

One of the earliest definitions of stress was published by Hans Selye (Selye, 1935), where stress was described as a “non-specific response or syndrome produced by diverse noxious agents such as exposure to cold, surgical injury, or excessive doses of a variety of drugs”. A more confusing description of stress was published more than 50 years later – “It seems as if stress, in addition to being itself and the result of itself, is also the cause of itself” (Jewell & Mylander, 1988). This latter definition recognises that the word “stress” can be used as a verb, noun or adjective, and therefore depending on the required context, stress can be defined as either a particular set of stimuli, the physiological state of the animal or the behavioural response.

Another definition achieves a distinction between the stressor and ensuing response – “stress is the recognition by the body of a stressor and therefore the state of threatened homeostasis; stressors are threats against homeostasis, and adaptive responses are the body’s attempt to counteract the stressor and re-establish homeostasis” (Chrousos *et al.*, 1988). The common theme of these definitions is the alteration to the body’s homeostasis, or resting state, following exposure to a noxious stimulus. Keeping this in mind, a concise definition of stress is a “state of threatened homeostasis”, and the stress response is the body’s attempt to regain and maintain homeostasis.

Noxious stimulation elicits a response known as the “general adaptation syndrome” that can be divided into three sequential stages (Selye, 1946):

1 – The initial “alarm reaction”, associated with activation of the peripheral sympathoadrenal system and hypothalamo-pituitary-adrenal (HPA) axis that generally occurs over a period of a few hours.

2 – A “stage of resistance”, where neuroendocrine signals from the sympathoadrenal system and HPA axis attempt to contain the stress.

3 – The final “stage of exhaustion”, where there is a gradual decline of stress resistance after repeated acute challenges or stimuli present for a number of days (i.e. chronic stress).

Even at this early stage of research into stress, the acute and chronic stress responses could be delineated. The acute “fight or flight” response that was first described by Cannon in 1929 (Cannon, 1929) has been conserved through multiple stages of evolution and is mediated primarily by the sympathoadrenal system. Activation of the sympathoadrenal system results in a rapid tachycardia and pressor response, and a redirection of blood from regions such as the skin and gastrointestinal tract to the voluntary muscles. These physiological changes can be achieved directly or indirectly via projections originating in the central nervous system (CNS) that may ultimately result in the release of the catecholamines such as adrenaline and noradrenaline (NA<sub>Dr</sub>). Studies have subsequently reported increased plasma levels of these catecholamines following various stressors (Marson *et al.*, 1989; Kvetnansky *et al.*, 1993).

Hormonal systems are also integral to the body’s stress response, with a considerable proportion of research investigating the response of different components of the HPA axis to a variety of stressors. These studies have reported elevated plasma levels of adrenocorticotrophic hormone (ACTH) and corticosterone in response to stimuli such as restraint and ether inhalation (McMurtry & Wexler, 1981; Chen & Herbert, 1995). Similar to the sympathoadrenal system, the release of corticotrophin-releasing factor (CRF), ACTH and corticosterone is controlled by the CNS. In particular, the paraventricular nucleus (PVN) of the hypothalamus has an integral role in the translation of neural information from the CNS into neuroendocrine signals that control the activity of the HPA axis. Neuronal cell bodies within the parvocellular PVN (pPVN) contain CRF and vasopressin, and these neurons project to the median eminence and anterior pituitary where they induce the release of ACTH (Gillies *et al.*, 1982; Swanson *et al.*, 1983). ACTH then acts as a secretagogue for corticosterone that is synthesised in the adrenal cortex (Bohus & de Kloet, 1981). The sensitivity of the neurons within the pPVN to various stressors has been well documented, with elevated levels of the mRNA encoding the immediate early gene (IEG) *c-fos* and its protein product Fos detected in the PVN of rats exposed to stressors such as restraint, demonstrating that neurons within this region are activated by stressful stimuli (Chen & Herbert, 1995; Cullinan *et al.*, 1995).

Many other physiological and hormonal systems are sensitive to the effects of noxious stimulation, with studies reporting alterations in the release of hormones such as insulin and testosterone during exposure to stressors such as restraint (Orr & Mann, 1990; Vargas *et al.*, 1994). The neuroendocrine and behavioural responses to stress represent the outputs occurring as a result of signals from the CNS and despite the range of physiological systems that are perturbed by stress, they all apparently act in synchrony to produce a united response to the stressor (e.g. Dallman *et al.*, 1995; Kvetnansky *et al.*, 1995). The stress response can be summarised in a concise network:



The inputs consist of the sensory cues, such as sight, smell, pain and sound that activate the CNS and initiate the central acute stress response. The CNS is responsible for perception of the stressor, where sensory signals are interpreted and if required, modified according to previous experiences. The final stage of the central stress response is the integration and translation of the perceived stressor into a physiological response.

The central stress response circuitry involves a complex network of nuclei from the autonomic control centres in the medulla to the integrative regions of the forebrain. Studies that map the distribution of *c-fos* mRNA, Fos-immunoreactivity (ir) or other markers of neuronal activation throughout the CNS of rats exposed to various stressors provide an indicator of the level of involvement of central regions in the stress response. Forebrain regions activated by stress include a variety of cortical subregions, including the orbitomedial, frontal (Fr), piriform (Pir) and cingulate (Cing) cortices, caudate putamen (CPu), thalamus, hypothalamus (PVN, dorsomedial hypothalamus (DM), supraoptic nucleus (SON)), amygdala, such as the medial subnucleus of the amygdala (Me), and hippocampus. Midbrain, pontine and medullary regions sensitive to stressful stimuli include the periaqueductal gray (PAG), parabrachial nucleus (PB), locus coeruleus (LC), raphe nuclei, rostral ventrolateral medulla (RVLM), caudal ventrolateral medulla (CVLM), area postrema (AP), nucleus of the solitary tract (NTS) and cerebellum (Melia *et al.*, 1994; Beck & Fibiger, 1995; Chen & Herbert, 1995; Cullinan *et al.*, 1995; Del Bel *et al.*, 1998; Emmert & Herman, 1999; Baffi & Palkovits, 2000; Chowdhury *et al.*, 2000).

Particular brain regions such as those above were shown to be sensitive to some, most or all types of stressors, leading some researchers to propose a theory of two distinct types of stressors based on the central pathways utilised for the production of the stress response (Herman &

Cullinan, 1997; Emmert & Herman, 1999). Psychological or neurogenic stressors activate "processive" pathways that involve higher brain centres such as the amygdala and hippocampus. Stressors such as restraint, novelty and fear do not pose an immediate threat to physiological homeostasis. Use of the processive circuitry permits integration and processing of the stressor, so that prior experience determines the magnitude of the response to the perceived stressor. It is these stressors that will be discussed mainly in the next section (section 1.1.1).

In contrast, physical stressors represent a real challenge to homeostasis, and include stimuli such as haemorrhage, exposure to ether and respiratory challenge. Physical stressors activate the so-called systemic pathway that directly stimulates central output regions such as the hypothalamus. The systemic pathway is thought to contain a lesser number of neuronal connections than the processive circuitry, as there is no requirement for higher order processing. This theory has been supported by investigations that have compared the pattern of *c-fos* mRNA expression in the CNS of rats exposed to processive and systemic stressors (Del Bel *et al.*, 1998; Emmert & Herman, 1999).

### 1.1.1 CHRONIC STRESS

Repeated exposure to a stressor often produces a response that is smaller in magnitude than the initial acute response. This phenomenon, firstly termed the final stage of exhaustion, has been called adaptation, habituation or coping behaviour. Adaptation has been observed at all levels of the body, ranging from the CNS, to physiological markers and finally to the behavioural response(s) elicited by the stressor. In the CNS, regions such as the hippocampus, PVN, amygdala (Me), septum and NTS express relatively lower levels of *c-fos* mRNA in chronically stressed rats when compared to rats acutely exposed to the same stressor (Melia *et al.*, 1994; Umemoto *et al.*, 1994; Chen & Herbert, 1995; Stamp & Herbert, 1999). The results of adaptation in the CNS are often manifested as changes in the activity of physiological systems. Compared to the peak levels detected during the acute stress response, reduced plasma levels of ACTH, corticosterone, adrenaline and NAdr have been observed in rats exposed to repeated restraint, swim and cold stress, although stressor intensity affects the level of adaptation (Konarska *et al.*, 1990a; Lachuer *et al.*, 1994; Stamp & Herbert, 1999).

Adaptive changes in the stress response have also been observed in the cardiovascular system. The peak tachycardic response does not differ markedly between acutely and

chronically stressed rats. Instead, it is the time required for this maximal tachycardia to return towards resting levels that is altered by repeated stress, with chronically stressed rats exhibiting the characteristic tachycardia followed by a rapid, dynamic fall in heart rate (HR) after a 10-15 minute period (Chen & Herbert, 1995; Stamp & Herbert, 1999). Stress-induced changes in body temperature follow a similar pattern, with the time required to regain normal levels decreasing as the number of stress sessions increase (Stamp & Herbert, 1999). Differences in stress-induced behaviours have also been observed in rats exposed to acute and chronic restraint, electroacupuncture and running (Bucinskaite *et al.*, 1996; Thorsell *et al.*, 1999).

Some researchers have investigated the occurrence of cross-tolerance to different stressors. More specifically, they have looked at how adaptation to one stressor affects the acute response to a new and different stressor. One study measured Fos expression in the CNS of rats that received an acute session of restraint after being exposed to intermittent cold stress for 7 days (Bhatnagar & Dallman, 1998). The results of the study indicate that rats exposed to chronic cold stress have an increased sensitivity to the restraint stressor. Interestingly, another study found that physiological alterations and improvements in the respiratory and cardiovascular systems associated with habituation to chronic restraint provided a better natural reaction and response to subsequent exposure to hypoxia (Meerson *et al.*, 1994). Moreover, other studies have reported that cross-tolerance is stressor-specific, with chronic exposure to restraint having no effect on the physiological response to ether inhalation (Terrazzino *et al.*, 1995).

## 1.2 RESTRAINT

Restraint is a robust psychological stressor that involves the containment of an animal in a perspex tube small enough to prevent the animal from turning around. This stress does not pose an immediate threat to homeostasis, but the novelty of the experience and the fear associated with being confined nevertheless activates the stress response. Familiarity with the protocol and surroundings following repeated exposure to the same paradigm often produces an adapted response (Chen & Herbert, 1995; Chowdhury *et al.*, 2000). Restraint may also be used to describe the stronger stress of immobilisation, where the animal is physically restrained or attached to a frame in an upright or supine position so that any movement is extremely difficult.

Comparison of the central stress response between rats exposed to restraint and immobilisation demonstrates that immobilisation is a much more stressful experience as indicated by elevated levels of *c-fos* mRNA/Fos-ir in the CNS of immobilised rats (Chowdhury *et al.*, 2000). In addition, increased levels of ACTH are observed in the plasma of immobilised rats compared to rats exposed to restraint (Campmany *et al.*, 1996).

Rats have been exposed to different periods of restraint, ranging from 2 min to 6 hours (Sakaguchi & Nakamura, 1990; Boone & McMillen, 1994a), but in general most authors restrain their rats for 30 min to 2 hours (Melia *et al.*, 1994; Chen & Herbert, 1995; Cullinan *et al.*, 1995; McDougall *et al.*, 2000). The neurochemical markers or physiological parameters that are under investigation govern the length of the restraint period. For instance, changes in HR and gene expression were evident after 2 min of restraint (Boone & McMillen, 1994a), abrogating the need for extended periods of restraint if HR or prepro-enkephalin (prepro-ENK) mRNA expression following acute stress is the sole focus of the study. However, immunohistochemistry often requires much longer time periods, sometimes waiting for 1 or 2 hours after cessation of the restraint stimulus to allow stress-induced protein production to achieve detectable levels. Extension of the restraint period for long periods of time is limited by habituation of the stress response (see section 1.2.1).

Many studies have used restraint as a psychological stressor and characterised various facets of the physiological and behavioural response. As seen with many stressors, the activity of the sympathoadrenal system in the rat is increased during exposure to restraint, with elevated plasma levels of adrenaline and NAdr reported by a number of studies (Kiritsy-Roy *et al.*, 1986; Marson *et al.*, 1989; Konarska *et al.*, 1990b). Moreover, catecholamine concentrations were found to be increased in regions of the rat brain that participate in sympathetic regulation (Lachuer *et al.*, 1991). The physiological consequences of elevated sympathetic activity include a rapid tachycardia, often increasing by as much as 200 beats per minute (bpm) in the first couple of minutes of restraint (Chen & Herbert, 1995; McDougall *et al.*, 2000). A pressor response to acute restraint that accompanies the tachycardia has also been reported in the rat (Barron & Van Loon, 1989; McDougall *et al.*, 2000).

Like all stressors, restraint activates the HPA axis. Increased synthesis of CRF mRNA has been detected in the pPVN following restraint, and is associated with an elevated release of CRF into the hypophysial portal circulation (Hashimoto *et al.*, 1989b; Harbuz *et al.*, 1994). The expression of the mRNA encoding pro-opiomelanocortin (POMC), the precursor for ACTH, is

increased in the anterior pituitary by CRF. As expected, it has been demonstrated that POMC mRNA levels were increased in the adenohypophysis of restrained rats (Larsen & Mau, 1994). Moreover, increased synthesis of POMC mRNA in the anterior pituitary during restraint was found to be translated into elevated concentrations of  $\beta$ -endorphin and ACTH, protein products of POMC (Hashimoto *et al.*, 1989b; Kjaer *et al.*, 1995a; Yamauchi *et al.*, 1997). Although  $\beta$ -endorphin belongs to the opioid family, it has been shown to depress pituitary portal plasma levels of CRF under resting conditions (Plotsky, 1986), while during restraint stress, Yamauchi and colleagues demonstrated that  $\beta$ -endorphin may potentiate the synthesis and/or release of ACTH (Yamauchi *et al.*, 1997). ACTH is well known as a potent stimulator of corticosterone release (Bohus & de Kloet, 1981). Elevated plasma corticosterone levels are widely accepted as an indicator of the level of stress in animals, and not surprisingly, many studies have reported an increase in plasma concentrations of corticosterone following restraint stress (Herbert & Howes, 1993; Ray *et al.*, 1993; Chen & Herbert, 1995; Stamp & Herbert, 1999; Thorsell *et al.*, 1999).

Vasopressin mRNA expression was also found to be elevated in the pPVN of rats exposed to restraint (Herman, 1995). Vasopressin is a potent stimulator of ACTH release in the adenohypophysis, and acts synergistically with CRF to activate the HPA axis during restraint (Gillies *et al.*, 1982). Interestingly, some studies have suggested that during chronic exposure to stressors such as restraint, vasopressin assumes a much more prominent role in the stimulation of ACTH release from the anterior pituitary (Scaccianoce *et al.*, 1991; Makino *et al.*, 1995). However, vasopressin released into the portal circulation during stress does not contribute significantly to global vasopressin plasma levels, with a number of studies reporting no change in peripheral plasma vasopressin concentration in rats exposed to restraint (Gibbs, 1984; Hashimoto *et al.*, 1989b; Kjaer *et al.*, 1995b).

The plasma concentration of testosterone was reduced in male rats exposed to acute restraint when compared to unstressed controls (Aloisi *et al.*, 1998). This gonadal hormone appears to interact with the HPA axis, as Viau and colleagues have demonstrated that testosterone can attenuate the release of vasopressin from the PVN (Viau & Meaney, 1996), and as a consequence, reduce the plasma levels of ACTH and corticosterone in restrained rats. Further investigation found that the medial preoptic area (POA) was a central region critical for this negative regulatory relationship between testosterone and the HPA axis.

The release of hormones other than those intrinsic to the HPA axis have also been altered by acute restraint. Plasma levels of prolactin and oxytocin are increased following exposure to a

restraint stress paradigm (Gibbs, 1984; Hashimoto *et al.*, 1989b; Larsen & Mau, 1994; Kjaer *et al.*, 1995b). Together with corticosterone and the catecholamines, prolactin and oxytocin assist in the mobilisation of glucose and energy stores for utilisation in the stress response (Dallman *et al.*, 1995). In contrast, insulin is a hormone that promotes energy storage, and considering this role, it was not surprising that plasma insulin levels were decreased by restraint (Vargas *et al.*, 1994; Makino *et al.*, 2000). Another anabolic hormone is testosterone, and as described in the previous paragraph, testosterone levels are also reduced by restraint.

Core body temperature has been the focus of a few studies investigating the physiological effects of restraint (Herbert & Howes, 1993; Chen & Herbert, 1995; Stamp & Herbert, 1999). These studies have observed a hypothermic response during acute exposure to restraint that is maximal within 10 minutes of the initiation of the stressor. As the restraint period continues, body temperature returns towards resting values. While swim stress was also found to produce a hypothermia, previously published studies have reported mainly increases in core body temperature following exposure to stressors such as a novel environment (Long *et al.*, 1989; Pavlovic *et al.*, 1996a). An additional study has found that restraint also produced a hyperthermic response, although it is unclear whether the increase in core temperature was due to the i.c.v. injections administered to the rats during the course of the experiment (Saiki *et al.*, 1997). However, some insights into the underlying mechanisms controlling stress-induced changes in body temperature have been reported. One early study suggested that restraint produced an inhibition of shivering thermogenesis, which consequently resulted in a fall in body temperature (Shimada & Stitt, 1983). Furthermore, peripheral (i.p.) administration of the non-selective opioid receptor antagonist, naloxone, in rats acutely exposed to restraint potentiated the drop in body temperature (Herbert & Howes, 1993), implicating the opioid system in this component of the physiological stress response.

Not surprisingly, restraint elicits many of the characteristic behaviours indicative of stressed animals. An increase in anxiety has been observed in rats exposed to restraint, as measured by behaviour in open field situations and the elevated plus maze (McBlane & Handley, 1994; Smagin *et al.*, 1996). Central regions contributing to restraint-induced anxiogenesis appeared to include the LC and amygdala (McBlane & Handley, 1994; Moller *et al.*, 1997). Inhibition of aggressive behaviour, freezing, and reduced exploratory behaviours have been observed in rats previously exposed to restraint (Albonetti & Farabollini, 1993; Albonetti & Farabollini, 1995; Zafar *et al.*, 1997). Restraint can also reduce food intake, with a larger effect observed in rats stressed at the beginning of the light cycle than in rats exposed to restraint at the conclusion of

the light cycle. Moreover, rats stressed in the morning still exhibited detrimental effects in feeding behaviour 2 hours into the dark cycle, with the underlying cause still awaiting further investigation (Rybkin *et al.*, 1997).

The level of nociception is influenced by different stressors, with a decrease in nociception (analgesia) often observed both during and after exposure to restraint (Kelly & Franklin, 1987; Gamaro *et al.*, 1998). Further experiments showed that restraint potentiated the analgesia produced by morphine and other more selective  $\mu$ -opioid agonists, therefore implicating  $\mu$ -opioid receptors in the stress response (Calcagnetti *et al.*, 1990; Calcagnetti *et al.*, 1992). The immune system is also affected by acute exposure to restraint. Farabollini and colleagues (Farabollini *et al.*, 1993) reported a reduction in mitogen-induced interferon- $\gamma$  production by splenocytes in restrained rats, suggesting that this particular stress does not stimulate lymphocyte or immunological activity. Blood levels of lymphocytes were reduced by restraint, and in addition, an increased sensitivity to immunological challenges has also been observed in restrained rats (Dhabhar *et al.*, 1994; Millan *et al.*, 1996).

### 1.2.1 CHRONIC RESTRAINT

As previously indicated, repeated exposure to the restraint paradigm results in adaptation and habituation at all levels of the stress response – central, neuroendocrine, physiological and behavioural. Within the CNS, chronic restraint caused a reduced expression of *c-fos* mRNA or Fos protein in various cortical structures and regions such as the hippocampus, PVN, amygdala (central nucleus (Ce) and Me), LC, septum and NTS (Melia *et al.*, 1994; Chen & Herbert, 1995; Stamp & Herbert, 1999). In addition, restraint has also resulted in alterations in a variety of neurochemical systems in specific central nuclei (see section 1.3). For example, expression of the transcript encoding the precursors for enkephalin (prepro-ENK) and neuropeptide Y (NPY) (prepro-NPY) in various brain regions was attenuated by repeated exposure to restraint when compared with the initial acute response (Boone & McMillen, 1994a; Thorsell *et al.*, 1999). Such data indicate the plasticity of the CNS in terms of integrating responses to stressors.

Neuroendocrine adaptation has been well documented, with chronic restraint producing an attenuated increase in ACTH and corticosterone release (Lachuer *et al.*, 1994; Chen & Herbert, 1995). Similarly, the restraint-induced rise in plasma catecholamine levels is reduced, and

sometimes non-existent in chronically stressed rats compared to rats exposed to the stressor on a single occasion (Konarska *et al.*, 1990b; Terrazzino *et al.*, 1995). In the cardiovascular system, adaptation was observed mainly within the initial restraint period, rather than during subsequent, additional exposures to the same stressor in normotensive rats (McDougall *et al.*, 2000). However, some studies have demonstrated that while the magnitude of the initial tachycardic response to restraint was maintained on subsequent exposures, the time required for HR to return to baseline levels was reduced by chronic restraint (Chen & Herbert, 1995). Furthermore, in rats where the cardiovascular system is compromised, as in Spontaneously Hypertensive (SHR) rats, adaptation of blood pressure (BP) and HR to chronic restraint was found to be attenuated when compared to normotensive rats (McDougall *et al.*, 2000) (see section 1.4.4).

Chronic restraint causes interesting effects on nociception. Compared to unstressed rats in the control group, chronically stressed rats had a lower threshold for pain (i.e. an increased level of nociception). Similarly, there was no stress-induced analgesia observed in chronically restrained rats; instead, a hyperalgesia was reported (Gamaro *et al.*, 1998). The reason for this response is presently unknown. Comparison of the hypothermic response of rats exposed to acute and chronic restraint revealed a similar temporal profile. However, the fact that restraint consistently produced a fall in body temperature during the first 10 minutes that returned towards resting levels as the restraint session continued suggests that habituation was occurring over this much shorter time span. General behavioural deficits, including increased depression as indicated by reduced exploratory activity in the open field, have been observed in Wistar-Kyoto (WKY) rats exposed to both acute (1 day) and chronic (8 days) restraint (Zafar *et al.*, 1997). Similarly, reductions in the number of leukocytes and lymphocytes induced by acute restraint were still evident after 11 days of restraint (Steplewski & Vogel, 1986). Almost 2 weeks after cessation of the restraint paradigm, some (but not all) immunological markers had returned to basal levels, demonstrating the long term effects of this particular psychological stressor (Steplewski & Vogel, 1986).

## 1.3 NEUROPEPTIDES

Techniques such as Fos immunohistochemistry, which provide a measure of the level of neuronal activation induced by a particular stimulus or stressor, provide a great deal of insight into which regions are implicated in the central stress response. However, these *c-fos*/Fos studies do have their shortcomings. While regions that are activated by a particular stimulus are easily identified, the lack of *c-fos* mRNA or Fos protein in a specific region does not necessarily mean that the region is not involved in the stress response. A reduction in baseline activity, which can be achieved through an increase in inhibitory afferent input, would not necessarily translate into detectable changes in *c-fos* mRNA or Fos-ir. Similarly, *c-fos*/Fos studies alone do not give any indication of the neurotransmitters/neuromodulators contributing to the stress response in the particular nucleus. Immunohistochemical studies using a combination of antibodies directed at Fos and a marker for a neurotransmitter such as a precursor, enzyme, receptor or the neurotransmitter itself can provide a great deal of information regarding the phenotype of activated neurons. Similarly, techniques such as *in situ* hybridisation histochemistry (ISHH) (see chapter 3), membrane binding and *in vitro* receptor autoradiography (see chapter 4) using specific probes and ligands can also contribute to a clearer understanding of the neurochemical changes occurring in specific brain regions during exposure to stress. Studies using these techniques have provided a significant body of evidence regarding stress-induced changes in numerous neuropeptides and neurotransmitters. The following section will focus on a number of neuropeptides and how different stressors, such as restraint, influence the synthesis of the neuropeptides and their precursors and the density of the pertinent receptors within the CNS. In addition, the following section also provides a relatively brief, general background on each neuropeptide, its localisation, the different receptor(s) that recognise the neuropeptide and their sensitivity to stressors.

### 1.3.1 OPIOIDS

#### 1.3.1.1 *Endogenous opioid peptide precursors*

The endogenous opioids are derived mainly from three known precursors - prepro-ENK (proenkephalin A), POMC, prodynorphin (proDYN or proenkephalin B) (Drouin & Goodman,

1980; Howells *et al.*, 1984; Civello *et al.*, 1985). These three precursors have been isolated and characterised as described below. While research into the related nociceptin/orphanin FQ peptides is still in its infancy, the lack of the characteristic C-terminal Tyr and an insensitivity to naloxone and  $\mu$ - $\delta$ - $\kappa$ -opioid receptor ligands separates it from the traditional opioid family of receptors (Meunier, 1997).

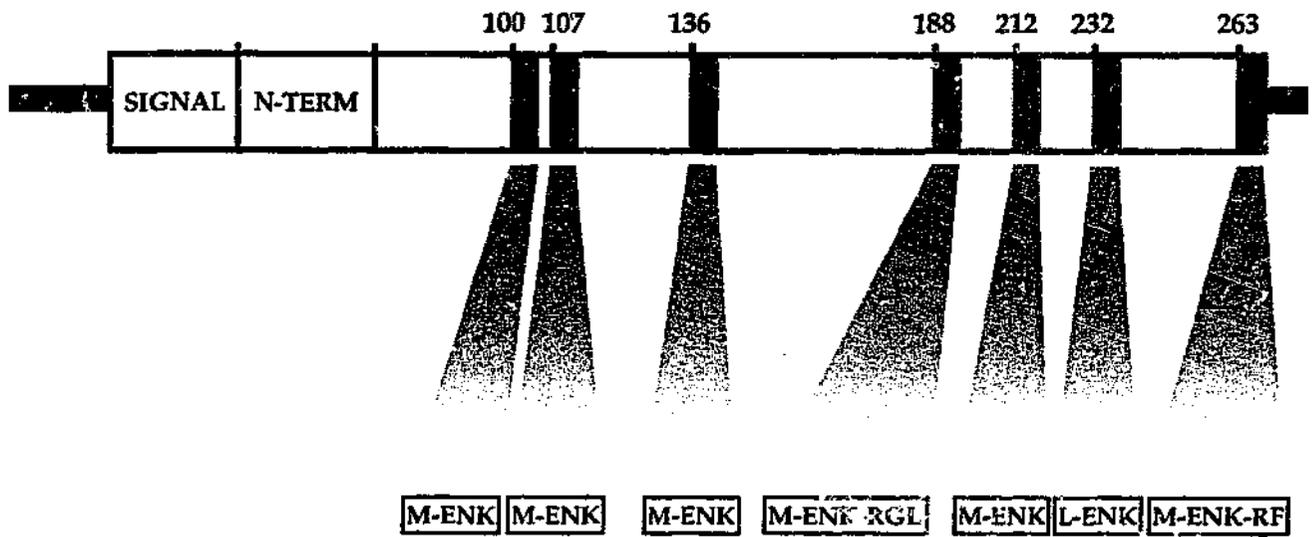
### *Prepro-ENK*

The rat prepro-ENK gene was isolated in 1984, with concurrent reports of the cloning and sequencing of rat prepro-ENK complementary DNA (cDNA) (Howells *et al.*, 1984; Rosen *et al.*, 1984; Yoshikawa *et al.*, 1984). In addition, the human prepro-ENK gene has also been cloned (Noda *et al.*, 1982) and localised to chromosome 8q23-q24 (Litt *et al.*, 1988). In the rat, the 5.3 kilobase (kb) prepro-ENK gene consists of 3 exons separated by 2 introns of approximately 600 base pairs (bp) and 3500 bp respectively. Once transcriptional processing has occurred, the remaining 269 amino acids that form the prepro-ENK peptide contain a 24 amino acid signal peptide and a 245 residue pro-ENK product (Figure 1.1) (Rosen *et al.*, 1984). Comparison of the amino acid sequence of rat prepro-ENK with the bovine and human gene products revealed an 82% homology with both species (Howells *et al.*, 1984). Further comparison of the sequence of prepro-ENK between these species demonstrated that the rat, human and bovine forms of prepro-ENK contain 4 copies of Met-enkephalin, and one copy each of Leu-enkephalin, Met-enkephalin-Arg<sup>6</sup>-Gly<sup>7</sup>-Leu<sup>8</sup> and Met-enkephalin-Arg<sup>6</sup>-Phe<sup>7</sup> (Howells *et al.*, 1984).

Detectable levels of prepro-ENK mRNA have been reported throughout the CNS and in peripheral tissues such as the heart, lymphocytes and testis (Howells *et al.*, 1986; Zurawski *et al.*, 1986; Yoshikawa & Aizawa, 1988). Using Northern blot analysis, brain regions containing prepro-ENK mRNA include the striatum, hypothalamus, midbrain, cerebellum, pons/medulla and spinal cord (Yoshikawa *et al.*, 1984). A much more detailed description of the central distribution of prepro-ENK mRNA has been reported by ISHH studies (Harlan *et al.*, 1987; Ahima *et al.*, 1992). This group found high levels of the prepro-ENK transcript in the cortex (Pir, neocortex, Cing, entorhinal (Ent), olfactory), nucleus accumbens (NAcc), olfactory tubercle, CPU, amygdala (Ce, Me), perifornical nucleus of the hypothalamus (PeF), ventromedial hypothalamus (VMH), cerebellum, PB, median raphe, raphe magnus (RMag), NTS, spinal trigeminal tract (sp5) and various levels of the spinal cord. Light to moderate levels of prepro-

**FIGURE 1.1**

Structure of prepro-ENK, together with the amino acid sequences of Met-enkephalin, the N-terminal extensions of Met-enkephalin and Leu-enkephalin. Note that all of the peptides shown contain the C-terminal YGGF sequence that is highlighted in blue and characteristic of opioid peptides.



Met-ENKEPHALIN



Met-ENKEPHALIN-RF



Met-ENKEPHALIN-RGL



Leu-ENKEPHALIN

ENK mRNA were also detected in the lateral septum, bed nucleus of the stria terminalis (BNST), other amygdaloid subregions, DM, PAG, LC, cuneate nucleus (Cu) and external cuneate (ECu).

Interestingly, the prepro-ENK gene contains a glucocorticoid response element in its regulatory region (Jenab & Inturrisi, 1995). Binding of glucocorticoids to this region may regulate the expression of prepro-ENK mRNA, with studies suggesting that circulating glucocorticoids are required to maintain basal prepro-ENK mRNA expression in regions such as the CPu, NAcc, VMH and Ce (Chao *et al.*, 1989; Ahima *et al.*, 1992). In addition, elevated levels of plasma glucocorticoids have induced significant increases in prepro-ENK mRNA expression in the CPu and hippocampus (Ahima *et al.*, 1992). These data suggest that prepro-ENK mRNA expression is extremely sensitive to plasma levels of glucocorticoids. This regulation of prepro-ENK mRNA becomes particularly important when plasma glucocorticoid concentrations are fluctuating such as during exposure to various stressors (see section 1.1).

### POMC

The structure of the rat gene encoding POMC was reported by Drouin and colleagues (Drouin *et al.*, 1985), with POMC genes also isolated and characterised in the human, cow and mouse (Nakanishi *et al.*, 1980; Takahashi *et al.*, 1981; Whitfield *et al.*, 1982; Uhler *et al.*, 1983). The rat POMC gene is approximately 6 kb long, and similar to the human, bovine and murine POMC genes, it contains 3 exons and 2 introns (Drouin *et al.*, 1985). Rat POMC mRNA has been found to exist in two forms, with two alternate splice locations in the 5' uncoding region (Oates & Herbert, 1984; Drouin *et al.*, 1985). As reported in earlier studies published by Oates (Oates & Herbert, 1984) and Drouin (Drouin & Goodman, 1980), the POMC precursor peptide consists of a 26 amino acid signal peptide and the 209 residue POMC prohormone. There are a number of different hormones contained in POMC, including  $\beta$ -lipotropin (91 residues),  $\gamma$ -melanocyte-stimulating hormone ( $\gamma$ -MSH; 12 residues),  $\alpha$ -MSH (13 residues),  $\beta$ -MSH (18 residues), ACTH (39 residues),  $\beta$ -endorphin (31 residues) and CLIP (corticotrophin-like intermediate lobe peptide; 22 residues) (Figure 1.2).

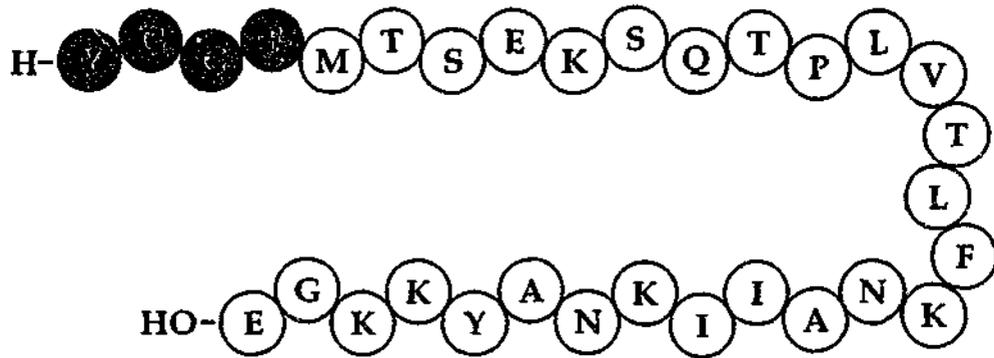
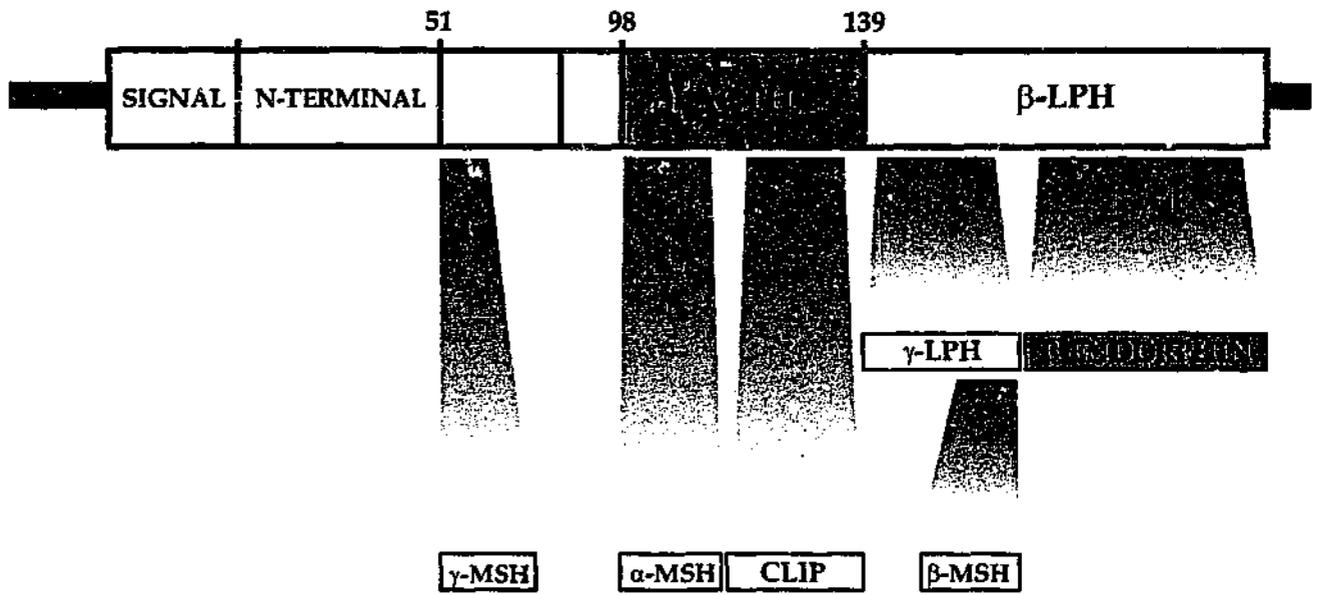
CRF and glucocorticoids such as corticosterone provide a mechanism that finely tunes the transcription of the POMC gene and subsequent synthesis of ACTH,  $\beta$ -endorphin and POMC-derived peptides. The rat POMC gene contains a CRF-responsive element, where CRF can promote POMC gene transcription and hence positively influence the production of ACTH (Jin

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**FIGURE 1.2**

Structure of POMC and the various peptides produced after enzymatic cleavage of POMC. The amino acid sequence of  $\beta$ -endorphin is also shown, with the characteristic YGGF C-terminal highlighted in blue.

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β-ENDORPHIN

*et al.*, 1994). In addition, a negative glucocorticoid regulatory element has been detected in the promoter region of the POMC gene, and binding of the glucocorticoid receptor to this region suppresses POMC gene synthesis (Drouin *et al.*, 1989). The activity of this glucocorticoid regulatory element appears to depend on the location. Adrenalectomy, where there is a rapid reduction in plasma corticosterone, induced an elevation in POMC mRNA levels in the anterior pituitary, while POMC mRNA in the intermediate lobe of the pituitary was not affected by fluctuations in plasma corticosterone concentrations (Pelletier, 1993; Baker & Herkenham, 1995). Contrasting reports have also been published regarding the regulation of POMC mRNA expression in the arcuate nucleus (ARC) in the hypothalamus (Beaulieu *et al.*, 1988; Pelletier, 1993; Baker & Herkenham, 1995), suggesting that further characterisation of this glucocorticoid-mediated modulation at the level of the ARC is required.

The central distribution of POMC mRNA in rats is extremely limited, with the only neuronal cell bodies containing POMC mRNA located in the hypothalamic ARC and in the NTS in the medulla oblongata (Finley *et al.*, 1981; Bronstein *et al.*, 1992; Larsen & Mau, 1994). However, neurons containing POMC-derived peptides originating from these regions project to many different nuclei, including the Ce, POA, VTA, PAG, NTS and median eminence (O'Donohue *et al.*, 1979; Gray *et al.*, 1984). POMC mRNA has also been detected in the intermediate and anterior lobes of the pituitary gland, ovary, testis, gastrointestinal tract, liver, kidney and spleen (Chen *et al.*, 1986; DeBold *et al.*, 1988).

### *ProDYN*

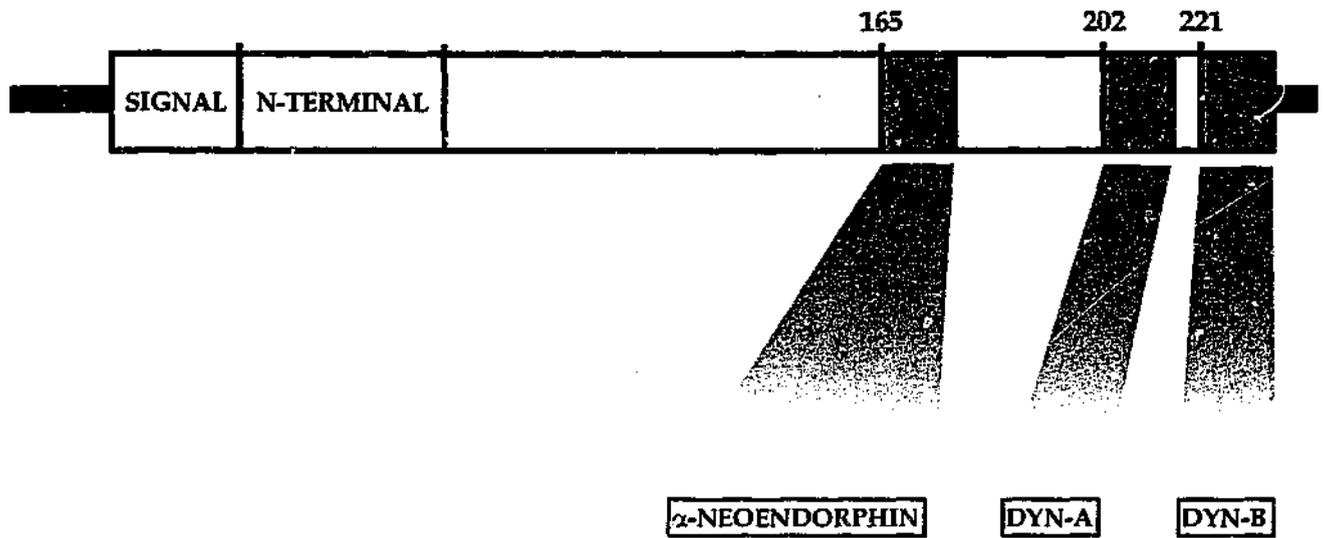
The first report of the characterisation of the rat proDYN gene was published in 1985 by Civello and colleagues (Civello *et al.*, 1985). This group focussed on the main exon of rat proDYN, and was able to determine a 204 amino acid sequence that codes for the majority of the translated region of rat proDYN. Within this 204 amino acid sequence are a number of biologically active opioid peptides, including Leu-enkephalin (3 copies), and  $\alpha$ -neoendorphin,  $\beta$ -neoendorphin, dynorphin A, dynorphin A (1-8) and dynorphin B (leumorphin) (Figure 1.3). In addition, the proDYN gene has been isolated in the human and localised to chromosome 20p12-pter, while the sequence of porcine proDYN mRNA has also been reported (Kakidani *et al.*, 1982; Horikawa *et al.*, 1983; Litt *et al.*, 1988). A glucocorticoid response element has not been identified in the proDYN gene; however, adrenalectomy decreased proDYN mRNA levels in the rat hippocampus, but not in the ARC (Thai *et al.*, 1992; Baker & Herkenham, 1995). These

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**FIGURE 1.3**

Structure of proDYN and its peptide products. The amino acid sequences of Leu-enkephalin,  $\alpha$ -neoendorphin, dynorphin A and dynorphin B are also shown, with the YGGF C-terminal sequence highlighted in blue in each peptide.

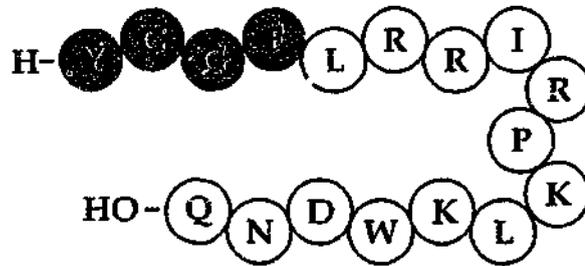
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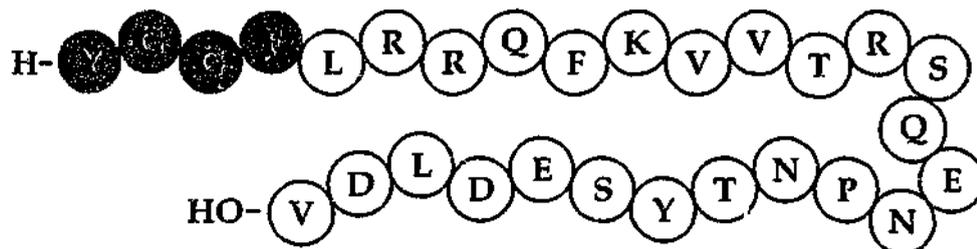
Leu-ENKEPHALIN



$\alpha$ -NEOENDORPHIN



DYNORPHIN A



DYNORPHIN B

results suggest that there may be region-specific selectivity in the effects of glucocorticoids on proDYN mRNA expression, but further studies are required to determine whether glucocorticoids achieve this regulation at the level of transcription.

The distribution of proDYN mRNA has been investigated using Northern blot analysis of RNA extracted from various brain regions and peripheral tissues (Civello *et al.*, 1985). In the rat CNS, high levels of proDYN mRNA were detected in the hypothalamus, CPu and hippocampus, with lower levels localised in the thalamus, cortex, midbrain, cerebellum, NTS and spinal cord. In the periphery, the highest levels of expression of proDYN mRNA were found in the adrenal gland, with proDYN mRNA also detected in the testis, anterior pituitary and ovary (Civello *et al.*, 1985; Kaynard *et al.*, 1992). The central distribution of proDYN mRNA has been studied in greater anatomical detail using ISHH. The highest densities of hybridisation signal to the proDYN probe were detected in neurons in the olfactory tubercle, NAcc, SON, PVN, CPu, Ce, dentate gyrus (DG), PB, Cu and NTS. In addition, various regions of the cortex and hypothalamus (DM, VMH, ARC, periventricular nucleus (Pe)), BM, Me, ventral pallidum, BNST, medial POA, PAG and LC expressed detectable levels of proDYN mRNA (Morris *et al.*, 1986; Mansour *et al.*, 1994b).

#### *Other opioid precursors*

Recently, the possible existence of additional opioid peptides has generated some interest. In 1995, Meunier and co-workers published the first report for a novel putative opioid ligand of 17 amino acids termed nociceptin, a peptide also referred to as orphanin FQ (Meunier *et al.*, 1995). Its classification as an "orphan" opioid peptide is due to its distinctive C-terminal sequence that does not contain the Tyr in position 1 that is characteristic of opioid peptides. The cDNA for nociceptin has been identified in the rat, as has the gene for the precursor in the rat, mouse and human (Mollereau *et al.*, 1996; Nothacker *et al.*, 1996). Novel putative opioid peptides (endomorphin-1 and -2) with a high selectivity for the  $\mu$ -opioid receptor have also been isolated (Zadina *et al.*, 1997), but the precursor for the endomorphins has not, as yet, been detected.

### 1.3.1.2 Synthesis and distribution of the endogenous opioids

The endogenous opioids can be grouped into 3 main families, the enkephalins, dynorphins, endorphins, and these have been extensively characterised in terms of their distribution and biological effects. However, additional opioid peptides, such as the endomorphins have also been postulated to have biological roles, and further research will provide a much clearer understanding of their contribution to the regulation of physiological systems and their associated neurochemistry.

#### *Enkephalins*

The enkephalin family consists primarily of Met-enkephalin and Leu-enkephalin, but carboxy terminal extensions of both pentapeptides (metorphamide, dynorphins, endorphin) have been shown to have pharmacological activity at different opioid receptor subtypes (Dray & Davis, 1985; Wang & Ingenito, 1994b). The enkephalins are obtained from two opioid precursors. Cleavage of prepro-ENK mRNA generates a number of enkephalin peptides (see section 1.3.1.1), while 3 copies of Leu-enkephalin are obtained from one proDYN peptide. Early investigations of the affinity of the enkephalins for the three main opioid receptor subtypes,  $\mu$ ,  $\delta$  and  $\kappa$  (see section 1.3.1.3), suggested that Met-enkephalin and Leu-enkephalin were the endogenous ligands for the  $\delta$ -opioid receptor subtype (Lord *et al.*, 1977). Since this study, the enkephalins have been reported to also bind to the  $\mu$ -opioid receptor subtype, while they do not bind appreciably to the  $\kappa$ -opioid receptor (Hawkins *et al.*, 1989; Nock *et al.*, 1990). Substituted enkephalin analogues that have been found to have a high selectivity for either the  $\mu$ - or  $\delta$ -opioid receptor subtype have been used extensively to study the role of both of these opioid receptor subtypes.

The central distribution of the enkephalins has been mapped by immunohistochemistry. Met-enkephalin-ir has been detected in perikarya and fibres in regions such as the lateral septal nucleus, BNST, the preoptic nuclei, hypothalamus (ARC, PVN, Pe, VMH, DM, mammillary nuclei), amygdala, hippocampus, cortex, ventral tegmental area (VTA), PAG, PB, LC and NTS (Wamsley *et al.*, 1980; Merchenthaler *et al.*, 1986; Sawchenko *et al.*, 1990).

### *Dynorphins*

The dynorphins, dynorphin A (1-17), dynorphin A (1-8) and dynorphin B (leumorphin), are C-terminal extensions of Leu-enkephalin that are obtained from the processing of proDYN (Figure 1.3). The dynorphins have been shown by numerous studies to have the highest affinity for the  $\kappa$ -opioid receptor subtype. Additionally, the dynorphins can bind to the  $\mu$ - and  $\delta$ -subtypes with considerable affinity (Chavkin *et al.*, 1982; Garzon *et al.*, 1984). Interestingly, one study has shown that the dynorphins can also bind to an NPY receptor (Miura *et al.*, 1994).

Using immunohistochemistry, the distribution of dynorphin-ir (dynorphin A, dynorphin B) has been described throughout the rat CNS, with cell bodies containing dynorphin-ir found in the NAcc, BNST, hippocampus, hypothalamus (SON, PVN, VMH), CPu, Ce, PAG, NTS and nucleus ambiguus (NAmb) (Molineaux *et al.*, 1982; Fallon & Leslie, 1986; Tan-No *et al.*, 1997). Dynorphin-ir positive fibres have also been visualised in the main efferent neuronal systems of these regions, including the striato-nigral, striato-pallidal, mossy fibre and hypothalamo-hypophysial pathways (Fallon & Leslie, 1986). In the periphery, dynorphin-ir has been detected in the pituitary, gastrointestinal tract, adrenal gland, kidney, heart and pancreas (Vincent *et al.*, 1984; Cetin, 1985; Bhargava *et al.*, 1988; Tan-No *et al.*, 1997). Dynorphin-ir has been detected in the CNS and peripheral tissues of species other than the rat, including the guinea pig and human (Gramsch *et al.*, 1982; Vincent *et al.*, 1984; Cetin, 1985).

### *$\beta$ -Endorphin*

As described in section 1.3.1.1, the precursor for  $\beta$ -endorphin is POMC. The POMC neurons are mainly localised in two populations, the ARC and NTS, and it is in these regions where the neuronal cell bodies containing  $\beta$ -endorphin-ir are predominantly found (Finley *et al.*, 1981; Bronstein *et al.*, 1992). In contrast to the limited distribution of  $\beta$ -endorphin positive cell bodies, terminals containing the  $\beta$ -endorphin peptide are found in numerous CNS regions. In the hypothalamus, PAG and NTS, a dense network of  $\beta$ -endorphin-ir terminals have been detected (Finley *et al.*, 1981).  $\beta$ -Endorphin-ir fibre networks have also been visualised in the PB, raphe nuclei, NAmb and the ventrolateral medulla (VLM) (Finley *et al.*, 1981; Schwartzberg & Nakane, 1983; Palkovits *et al.*, 1987). In the rat, peripheral tissues such as the gastrointestinal tract, pituitary, testis, kidney, liver, heart, adrenals, lung and spleen contain detectable levels of  $\beta$ -endorphin (Bhargava *et al.*, 1988; DeBold *et al.*, 1988).

### *Other endogenous opioids*

Included in this additional group of putative endogenous opioids are the recently discovered peptides such as the endomorphins. The endomorphins have been characterised by their high affinity for the  $\mu$ -opioid receptor, and have been found in high concentrations in central regions known to have dense populations of  $\mu$ -opioid receptors (Zadina *et al.*, 1997; Schreff *et al.*, 1998). Nociceptin binds primarily to the orphan receptor, with little or no affinity for the  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors. This selectivity may be explained by the substitution of a Phe amino acid for the initial Tyr that is normally present in position 1 at the amino end of opioid peptides (Meunier *et al.*, 1995). In addition, the fact that the functions and effects mediated by nociceptin are insensitive to naloxone isolates nociceptin from the traditional opioid receptor family.

#### *1.3.1.3 Opioid receptors*

As mentioned in the sections above, there are 3 main opioid receptor subtypes ( $\mu$ ,  $\delta$  and  $\kappa$ ), all of which belong to the seven transmembrane domain G-protein-coupled receptor family. These receptors will be discussed in the following section, in terms of their distribution and second messenger systems.

#### *$\mu$ -opioid receptor*

The rat  $\mu$ -opioid receptor was cloned in 1993 (Chen *et al.*, 1993a; Fukuda *et al.*, 1993; Thompson *et al.*, 1993), and subsequent studies have isolated and characterised  $\mu$ -opioid receptors in the human and mouse (Min *et al.*, 1994; Wang *et al.*, 1994a). The human  $\mu$ -opioid receptor is approximately 95% homologous to the rat  $\mu$ -opioid receptor, and has been localised to chromosome 6q24-25 (Wang *et al.*, 1994a). Pharmacological studies have suggested that there may be two subtypes of the  $\mu$ -opioid receptor ( $\mu_1$  and  $\mu_2$ ), but at present only one receptor has been cloned (Wolozin & Pasternak, 1981; Thompson *et al.*, 1993). Interestingly, two splice variants of the  $\mu$ -subtype have been isolated that differ in their sensitivity to agonist-induced sensitisation and internalisation (Zimprich *et al.*, 1995; Koch *et al.*, 1998). The two isoforms exhibit similar binding characteristics, suggesting that these splice variants do not represent individual  $\mu_1$  and  $\mu_2$  receptors (Zimprich *et al.*, 1995; Koch *et al.*, 1998). However, a recent

study has used immunohistochemistry to demonstrate distinct central distributions of multiple  $\mu$ -opioid receptor splice variants (Abbadie *et al.*, 2000).

The distribution of the mRNA encoding the  $\mu$ -opioid receptor has been reported in a number of studies. Using ISHH, Mansour and colleagues (Mansour *et al.*, 1994c) found that  $\mu$ -opioid receptor mRNA was distributed throughout many levels of the rat CNS. High levels of  $\mu$ -opioid receptor mRNA were detected in neurons in the olfactory bulb (OB), accessory OB, olfactory nuclei, CPu, NAcc, globus pallidus, ventral pallidum, septum, BNST, amygdala (intercalated, Me, cortical), hippocampus, most thalamic nuclei, medial preoptic nucleus, posterior hypothalamic nucleus, median raphe, RMag, LC, PB, NAmb, AP, NTS, Cu, dorsal motor nucleus of the vagus (DMX) and spinal cord. Lower levels of the  $\mu$ -opioid receptor transcript are localised in neurons in regions such as the amygdala (Ce, lateral, basolateral (BL), basomedial (BM)), hypothalamus (ARC, DM), PAG and VTA. Note that there was no discernable  $\mu$ -opioid receptor mRNA detected in the PVN or SON (Thompson *et al.*, 1993; Mansour *et al.*, 1994c). In addition,  $\mu$ -opioid receptor mRNA has been detected in glial cell membranes in regions such as the cortex, hypothalamus and striatum, although the transcript for  $\delta$ - and  $\kappa$ -opioid receptors were more abundant (Ruzicka *et al.*, 1995).

Generally, there appears to be a good correlation between the distribution of  $\mu$ -opioid receptor mRNA and  $\mu$ -opioid receptors visualised using ISHH and autoradiography, respectively. In the past, different  $\mu$ -selective radiolabelled ligands have been used to investigate the central distribution of  $\mu$ -opioid receptors, including [ $^3$ H]-DAMGO (DAGO; Tyr-D-Ala-Gly-MePhe-Gly-ol), [ $^3$ H]-CTOP ([ $^3$ H]-H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub>) and [ $^{125}$ I]-FK 33-824 [ $^{125}$ I]-D-Ala<sup>2</sup>-Me-Phe<sup>4</sup>-met(o)-ol]-enkephalin (Rothman *et al.*, 1987; Hawkins *et al.*, 1988; Mansour *et al.*, 1994c; Cowen *et al.*, 1999). These studies have detected highest densities of  $\mu$ -opioid receptors in neurons in cortical regions such as the Cing, Par, and temporal cortex, NAcc, CPu, septum, stria terminalis (st), thalamus, hippocampus, DG, amygdala (Me, cortical, lateral, BL), substantia nigra (pars compacta), median raphe, LC, PB, NAmb, AP, NTS, DMX and spinal cord (Hawkins *et al.*, 1988; Mansour *et al.*, 1994c). Regions where neuronal  $\mu$ -opioid receptors have also been detected include the globus pallidus, subfornical organ, hypothalamus (DM, lateral hypothalamus (LHy)), VTA and PAG (Rothman *et al.*, 1987; Hawkins *et al.*, 1988; Mansour *et al.*, 1994c; Cowen *et al.*, 1999).

Activation of  $\mu$ -opioid receptors expressed in COS-7 and Chinese Hamster Ovary (CHO)-K1 cells has been shown to inhibit the activity of adenylate cyclase via a pertussis toxin sensitive mechanism (Chen *et al.*, 1993a; Zimprich *et al.*, 1995). The sensitivity to pertussis toxin suggests that the  $\mu$ -opioid receptor may couple to the inhibitory  $G_i/G_o$  proteins, and a recent review describes the many different G protein subunits that have been associated with the  $\mu$ -opioid receptor effector pathway (Connor & Christie, 1999). Moreover, stimulation of  $\mu$ -opioid receptors inhibited N-type calcium ( $Ca^{2+}$ ) currents and activated inwardly-rectifying potassium ( $K^+$ ) channels (Loose & Kelly, 1990; Schroeder *et al.*, 1991). Interestingly, activation of  $\mu$ -opioid receptors resulted in a biphasic effect on  $K^+$  conductance in dorsal root ganglion (DRG) cells that is concentration-dependent and may involve different G-protein subunits (Fan & Crain, 1995).

### *$\delta$ -opioid receptor*

The first opioid receptor to be cloned was the murine  $\delta$ -opioid receptor in 1992 by Evans and colleagues (Evans *et al.*, 1992). The murine  $\delta$ -opioid receptor cDNA then became a valuable tool in isolating not only the rat  $\delta$ -opioid receptor cDNA, but also the rat  $\mu$ -opioid receptor (Chen *et al.*, 1993a; Fukuda *et al.*, 1993). The rat and murine  $\delta$ -opioid receptors are 97% homologous, while the rat  $\delta$ - and  $\mu$ -opioid receptors share a 61% homology (Fukuda *et al.*, 1993). The cloning of the rat and murine  $\delta$ -opioid receptors ultimately led to the isolation of the human cDNA for the  $\delta$  receptor (Knapp *et al.*, 1994; Simonin *et al.*, 1994). Furthermore, the human  $\delta$ -opioid receptor has been localised to chromosome 1p34.3-p36.1 (Befort *et al.*, 1994).

The distribution of the  $\delta$ -opioid receptor subtype throughout the rat CNS has been reported, and it was found to be distinct from both the  $\mu$ - and  $\kappa$ -opioid receptors. High levels of the mRNA encoding the  $\delta$ -opioid receptor have been detected in neurons in the anterior olfactory nucleus, cortex (Cing, Ent, Fr, Par, prefrontal, temporal), CPU, NAcc, olfactory tubercle, hippocampus, DG, amygdala (BL, BM, lateral, Me), claustrum, VMH, red nucleus, trigeminal nuclei, ECu, nucleus of the trapezoid body (Tz), hypoglossal nucleus (XII) and prepositus hypoglossi (PrH), cerebellum and spinal cord. Low to moderate levels of  $\delta$ -opioid receptor mRNA have been observed in the BNST, dorsal endopiriform nucleus (DEN), thalamus, PVN, SON, superior colliculus, substantia nigra and NTS (Mansour *et al.*, 1994a). Glial cell membranes also contain detectable levels of  $\delta$ -opioid receptor mRNA in regions such as the

cortex, striatum, cerebellum and hypothalamus (Ruzicka *et al.*, 1995). Peripherally, the  $\delta$ -opioid receptor has been found in the gastrointestinal tract, adrenal gland, kidney, liver, heart, lungs, spleen, testis, ovary and uterus (Wittert *et al.*, 1996).

Autoradiographic studies of the  $\delta$ -opioid receptor in the rat CNS have utilised many different ligands, such as [ $^3$ H]-Naltrindole, [ $^3$ H]-D-Ala<sup>2</sup>-D-Leu<sup>5</sup>-Enkephalin (DADLE/DADL), [ $^3$ H]-[D-Pen<sup>2</sup>,D-Pen<sup>5</sup>]enkephalin (DPDPE) and [ $^3$ H]-D-Ser<sup>2</sup>-Leu<sup>5</sup>-Thr<sup>6</sup>-Enkephalin (DSLET). Using either [ $^3$ H]-naltrindole, a highly selective  $\delta$ -opioid receptor antagonist, or the agonist [ $^3$ H]-DPDPE, high densities of  $\delta$  receptors have been identified in the OB, cortex, CPu, NAcc, hippocampus, DG, ventral medial thalamus, DEn, claustrum, amygdala (BL, lateral, Me), olfactory tubercle and st.  $\delta$ -Opioid receptors were also detected in the septum, habenula (Hab), globus pallidus, hypothalamus, inferior olivary complex (IOC) and spinal cord (Mansour *et al.*, 1987; Drower *et al.*, 1993). When the central  $\delta$ -opioid receptor distribution and density was compared using [ $^3$ H]-DPDPE or [ $^3$ H]-DSLET as ligands, significant differences in density were detected in a number of regions such as the cortex, CPu, NAcc, globus pallidus, BNST, hippocampus, amygdala, hypothalamus (DM, VMH) and PAG (Hiller *et al.*, 1996). [ $^3$ H]-DSLET binding site density was elevated in all of the above areas, and [ $^3$ H]-DSLET binding sites have been postulated to represent the " $\delta_2$  subtype". Despite pharmacological evidence such as this suggesting that two  $\delta$ -opioid receptor subtypes exist, only one  $\delta$ -opioid receptor has been cloned, and researchers await further biochemical data before confirming the presence of distinct  $\delta_1$  and  $\delta_2$  opioid receptors. Recently, reports have demonstrated that the opioid receptors may dimerise, with  $\delta$ -opioid receptors forming homodimers (Cvejic & Devi, 1997) and heterodimers with  $\kappa$ -opioid receptors (Jordan & Devi, 1999). Future studies into the isolation and characterisation of opioid receptor dimers may contribute to a better understanding of the physiological effects of opioid receptor stimulation.

The signal transduction mechanisms of the  $\delta$ -opioid receptor have been extensively studied, and are similar to those of the  $\mu$ -opioid receptor.  $\delta$ -Opioid receptors are coupled to the G<sub>i</sub>/G<sub>o</sub> subtypes of G proteins, as stimulation of the  $\delta$ -opioid receptor inhibited accumulation of cyclic 3',5'-adenosine monophosphate (cAMP) and this functional effect was blocked by addition of pertussis toxin (Law *et al.*, 1985). Activation of  $\delta$  receptors inhibited Ca<sup>2+</sup> currents through blockade of N-type Ca<sup>2+</sup> channels (Hescheler *et al.*, 1987), and a biphasic effect on K<sup>+</sup> channel conductance similar to the  $\mu$ -opioid receptor has also been observed (Fan & Crain, 1995).

*κ-opioid receptor*

The dynorphins are known to have a high affinity for the  $\kappa$ -opioid receptor (Chavkin *et al.*, 1982), and the cDNA and gene encoding this opioid receptor have been isolated and characterised in the rat (Chen *et al.*, 1993b; Li *et al.*, 1993; Yakovlev *et al.*, 1995). In terms of homology, the rat  $\kappa$ -opioid receptor has a 60% amino acid similarity to the rat  $\mu$  receptor, and is 59% homologous to the rat  $\delta$  receptor (Li *et al.*, 1993). The mouse and human genes encoding the  $\kappa$ -opioid receptor have also been cloned (Nishi *et al.*, 1994; Simonin *et al.*, 1995), with the human  $\kappa$ -opioid receptor localised to chromosome 8q11-12 (Simonin *et al.*, 1995).

Variations in the affinity of selective ligands for the  $\kappa$ -opioid receptor suggests that at least 3 subtypes of the  $\kappa$ -opioid receptor may exist (Zukin *et al.*, 1988; Cheng *et al.*, 1992), while Yakovlev and colleagues identified a splice variant of the  $\kappa$ -opioid receptor (Yakovlev *et al.*, 1995). However, the localisation of this additional transcript does not correlate well with the expected tissue distribution for the three putative  $\kappa$ -opioid receptors. Moreover, the fact that only one  $\kappa$ -opioid receptor gene has been cloned suggests that additional  $\kappa$ -opioid receptors may not exist, and the variations in binding affinity observed by Zukin (Zukin *et al.*, 1988) and Cheng (Cheng *et al.*, 1992) may be a simple delineation of different conformational states of the  $\kappa$ -opioid receptor. Additionally, the recently isolated heterodimer consisting of  $\delta$ - and  $\kappa$ -opioid receptors may also contribute to these affinity differences observed in the  $\kappa$ -opioid receptor family (Jordan & Devi, 1999). Consequently, considerable pharmacological and molecular biological research is still required to provide a better understanding of the  $\kappa$ -opioid receptor family.

Following the cloning of the  $\kappa$ -opioid receptor and determination of the sequence of the mRNA encoding the receptor, oligonucleotides were synthesised and used to investigate the anatomical distribution of the  $\kappa$ -opioid receptor. Dense populations of neurons expressing  $\kappa$ -opioid receptor mRNA have been localised in cortical nuclei such as the orbital, insular, Par, Ent and temporal cortex, claustrum, DEn, NAcc, olfactory tubercle, CPu, amygdala (Ce, BL), BNST, medial POA, paraventricular thalamic nucleus (PVA), hypothalamus (PVN, SON, DM), VTA, substantia nigra, PAG and NTS (Mansour *et al.*, 1994b). Low to moderate levels of the  $\kappa$ -opioid receptor transcript were detected in neurons in the OB, lateral septum, ventral pallidum, globus pallidus, amygdala (Me, BM, lateral), DG, various thalamic nuclei, hypothalamus (ARC, VMH), raphe nuclei, PB, LC and PrH (Mansour *et al.*, 1994b). Regions such as the cerebellum, cortex

and hypothalamus contain detectable levels of  $\kappa$ -opioid receptor mRNA in glial cell membranes (Ruzicka *et al.*, 1995).

As is the case with the rat  $\mu$ - and  $\delta$ -opioid receptors, there are a number of commercially available radioligands that can label the  $\kappa$ -opioid receptor, including [ $^3\text{H}$ ]-bremazocine, [ $^3\text{H}$ ]-ethylketocyclazocine ([ $^3\text{H}$ ]-EKC) and [ $^3\text{H}$ ]-U69-593 ((5a,7a,8b)-(+)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)-benzeneacetamide) (Morris & Herz, 1986; Tempel & Zukin, 1987; Mansour *et al.*, 1994b). Further characterisation of the binding affinity of these ligands found that some of these were not as selective as first thought, with [ $^3\text{H}$ ]-bremazocine and [ $^3\text{H}$ ]-EKC labelling additional opioid receptor subtypes. Thus, [ $^3\text{H}$ ]-U69-593 and U50-488H became more widely used as  $\kappa$ -selective ligands than the non-selective bremazocine and EKC (Zukin *et al.*, 1988; Nock *et al.*, 1990). Using [ $^3\text{H}$ ]-U69-593,  $\kappa$ -opioid receptors have been visualised in the claustrum, DEn, NAcc, CPu, lateral septum, amygdala (BL, BM, Me), BNST, st, thalamus (PVA, zona incerta (ZI)), hypothalamus (PVN, Pe, SON, VMH), PAG, LC, PB, PrH and NTS (Mansour *et al.*, 1994b).

During the functional and autoradiographic studies of the  $\kappa$ -opioid receptor, it was discovered that there was a population of U69-593-insensitive  $\kappa$ -opioid receptors that also existed. Mansour and colleagues (Mansour *et al.*, 1994b) compared the distribution of [ $^3\text{H}$ ]-U69-593 binding sites and [ $^3\text{H}$ ]-bremazocine binding sites in the presence of DAMGO and DPDPE. They reported clearly lower levels of [ $^3\text{H}$ ]-U69-593 binding sites in regions such as the OB, dorsal hippocampus, DG, some thalamic nuclei and the cerebellum (Mansour *et al.*, 1994b). Further autoradiography experiments using the non-selective ligands [ $^3\text{H}$ ]-bremazocine or [ $^3\text{H}$ ]-EKC in the presence of unlabelled U69-593 and  $\mu$ - and  $\delta$ -blocking ligands provided further identification of U69-593-insensitive  $\kappa$ -opioid receptors (Nock *et al.*, 1993). These results were not only found in the rat, with Nock and colleagues also revealing that this additional receptor with a high affinity for bremazocine and EKC was present in the guinea pig, cow, pig and chicken (Nock *et al.*, 1993). Thus, these studies led researchers to classify two separate  $\kappa$ -opioid receptors, the  $\kappa_1$  (U69-593-sensitive) and  $\kappa_2$  (U69-593-insensitive), although only one  $\kappa$ -opioid receptor has been cloned at present. Nock and colleagues (Nock *et al.*, 1993) have also postulated that the putative  $\kappa_2$  receptor may also be the so-called  $\epsilon$ -opioid receptor.

Like all opioid receptors, the  $\kappa$ -opioid receptor has been shown to exert its cellular effects through G protein-linked second messengers. Chen (Chen *et al.*, 1993b) and Zhang (Zhang &

Wong, 1998) reported that addition of  $\kappa$ -selective agonists to COS-7 cells transfected with the  $\kappa$ -opioid receptor or cardiac myocytes resulted in a reduction of adenylate cyclase activity that could be blocked by pertussis toxin, suggesting an involvement of  $G_i/G_o$  G protein subunits. Moreover, an elevation in intracellular  $Ca^{2+}$  concentration has been observed following stimulation of  $\kappa$ -opioid receptors in cardiac myocytes (Zhang & Wong, 1998).

#### 1.3.1.4 Opioids and stress

The opioids have been implicated in many components of the physiological and behavioural response to a variety of stressors. Exposure to physical stressors such as hypoglycaemia, haemorrhage and hypoxia results in alterations in precursor and peptide levels in the rat CNS (Morley *et al.*, 1982; Fan & McIntosh, 1993b; Johnson *et al.*, 1994; Tkacs *et al.*, 2000). Furthermore, chronic salt loading and i.p. injection of hypertonic saline increases proDYN and prepro-ENK mRNA expression in the magnocellular neurons of the rat PVN and SON (Meister *et al.*, 1990a; Young & Lightman, 1992; Yagita *et al.*, 1994). Functional reports have also provided strong support for a role for opioids in the central and neuroendocrine response to physical stressors such as water deprivation, hypertonic saline administration and hypovolaemia (Summy-Long *et al.*, 1981; Ukai & Holtzman, 1988; Munro *et al.*, 1994; Bodnar *et al.*, 1995).

Psychological stressors can also elicit changes in central opioid neurochemistry, as measured by changes in the expression of mRNA encoding the opioid precursors. Prepro-ENK and POMC mRNA expression were elevated following exposure to stressors such as social deprivation and restraint in regions of the rat CNS such as the PVN, NTS, RVLM and CVLM (Iglesias *et al.*, 1992; Boone & McMillen, 1994a; Harbuz *et al.*, 1994; Larsen & Mau, 1994). Boone and colleagues (Boone & McMillen, 1994a) investigated the time course and plasticity of prepro-ENK mRNA expression in the medulla oblongata of rats subjected to a 2 min and 30 min restraint paradigm. The results of the study demonstrate that in the AP, but not in the NTS, CVLM or RVLM, prepro-ENK mRNA levels were sensitive to the duration of the stressor, even within the short 30 min restraint period. Thus, the neurons in the AP that contain prepro-ENK mRNA exhibited a degree of plasticity during exposure to restraint.

The changes in expression of the opioid precursors induced by psychological stress have apparently been translated into significant alterations in opioid peptide levels in various central regions. In the hypothalamus, social stress increased Met-enkephalin-ir, while a chronic swim

stress induced a significant decrease in  $\beta$ -endorphin-ir in the rat (Iglesias *et al.*, 1992; Bidzinska *et al.*, 1993). In addition, restraint increased  $\beta$ -endorphin-ir in the rat PAG 24 hours after cessation of the stimulus (Farabollini *et al.*, 1993). Alterations in receptor density may serve as additional neurochemical markers indicating sensitivity of the central opioid system to psychological stress. As such,  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptor density in the rat CNS has been significantly increased or decreased following exposure to a psychological stressor. For example, Zeman and colleagues have reported increased  $\mu$ - and  $\delta$ -opioid receptor density in the CPU of rats exposed to immobilisation, while septal  $\mu$ -opioid receptor density decreased after exposure to a footshock paradigm (Zeman *et al.*, 1988; Stein *et al.*, 1992).

Central administration of opioid agonists or antagonists prior, or during exposure, to psychological stress have provided an insight into the opioid-mediated regulation of the physiological or behavioural stress response. The opioids have been implicated in the cardiovascular response to psychological stress, and reports have been published regarding their role. Some studies have reported that systemic (i.p.) or intra-PVN naloxone had no effect on the tachycardic response to restraint (Kiritsy-Roy *et al.*, 1986; Herbert & Howes, 1993), while others demonstrated that central (i.c.v.) or systemic (i.p.) administration of naloxone in rats suppressed the elevation in BP caused by different psychological stressors, including cold, intense light and sound, footshock and isolation (Florentino *et al.*, 1987; Jiménez *et al.*, 1990). Interestingly, microinjection of the selective  $\mu$ -opioid receptor agonist DAGO into the lateral ventricle or PVN suppressed the restraint-induced tachycardia through a modulation of parasympathetic activity (Kiritsy-Roy *et al.*, 1986; Marson *et al.*, 1989).

Changes in sympathetic outflow can be manifested as changes in BP, as well as alterations in plasma levels of the catecholamines during exposure to stress. The opioids have been implicated in the regulation of catecholamine secretion during stress, as systemic naloxone significantly inhibited the restraint-induced secretion of adrenaline and NAdr in conscious rats (Yamauchi *et al.*, 1997). While  $\beta$ -endorphin may not be involved in the modulation of the sympathoadrenal axis (Yamauchi *et al.*, 1997), Marson and co-workers demonstrated that stimulation of central  $\mu$ -opioid receptors with i.c.v. DAGO potentiated the elevated secretion of catecholamines in response to restraint (Marson *et al.*, 1989). An earlier study by Kiritsy-Roy and colleagues (Kiritsy-Roy *et al.*, 1986) found that microinjection of the non-selective opioid antagonist naloxone into the PVN of rats exposed to restraint potentiated the elevated plasma levels of adrenaline, but not NAdr. However, in the same study, direct stimulation of  $\mu$ -opioid receptors

in the PVN with DAGO had no effect on catecholamine secretion, suggesting that other opioid receptor subtypes may be involved in the regulation of sympathetic outflow at the level of the PVN (Kiritsy-Roy *et al.*, 1986).

Another primary component of the physiological stress response is the HPA axis, and evidence supports a modulatory relationship between the opioid system and the HPA axis. Studies have detected a stimulatory effect of opioids on stress-induced activation of the HPA axis, with  $\beta$ -endorphin, dermorphin (a  $\mu$ -opioid receptor agonist) and Met-enkephalin increasing the secretion of ACTH and corticosterone in the rat in response to restraint or a swimming stress (Herbert & Howes, 1993; Degli Uberti *et al.*, 1995; Gadek-Michalska *et al.*, 1997a; Yamauchi *et al.*, 1997). The  $\kappa$ -opioid receptor may oppose these actions, as i.p. administration of the  $\kappa$ -opioid receptor agonist ketocyclazocine suppressed the secretion of ACTH in response to a cold-restraint stress paradigm (Ray *et al.*, 1993).

Evidence demonstrates that opioids can regulate the release of a variety of hormones. Studies have shown that the release of vasopressin and oxytocin can be inhibited by peripheral (s.c. or i.v.) and central (i.c.v.) administration of  $\mu$ - and  $\kappa$ -, but not  $\delta$ -opioid receptor agonists, while i.v. naloxone reduced plasma levels of prolactin in the unstressed rat (Xu & McCann, 1989; Van de Heijning *et al.*, 1991; Ludwig *et al.*, 1997). During exposure to psychological stressors, the release of these hormones is also under the modulatory influence of the central opioid system. Naloxone and the  $\kappa$ -opioid antagonist MR2266 BS, both administered peripherally (s.c.), have elevated the restraint- and hypertonic saline-induced secretion of oxytocin (Carter & Lightman, 1987), while  $\mu$ - and  $\kappa$ -opioid receptor selective antagonists inhibit the stress-induced release of prolactin (Petraglia *et al.*, 1987; Xu & McCann, 1989). In addition to the neuroendocrine limb of the stress response, the opioids also appear to have a role in the regulation of body temperature, analgesia and motor suppression during stress (Chipkin *et al.*, 1982; Nabeshima *et al.*, 1985; Calcagnetti *et al.*, 1990; Herbert & Howes, 1993).

## 1.3.2 GALANIN

### 1.3.2.1 *Synthesis and distribution of GAL*

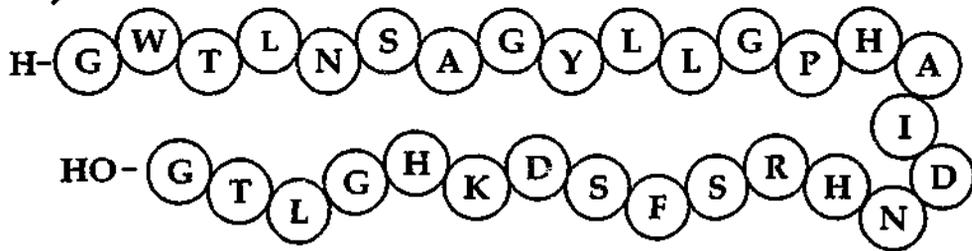
Galanin (GAL) was initially isolated from the porcine intestine by Tatemoto and colleagues in 1983 (Tatemoto *et al.*, 1983), and the peptide was named after the N-terminal glycine and C-terminal alanine. The presence of GAL has been reported in a number of species, including humans (Gentleman *et al.*, 1989), insects (Lundquist *et al.*, 1993) and invertebrates (Diaz-Miranda *et al.*, 1996). The GAL peptide sequence has been reported in at least 15 different species, and in the majority of these species it exists as a 29 amino acid peptide which is amidated at the C-terminal. It is only in the human where GAL has been reported to exist as a peptide of 30 amino acids, and it is not C-terminally amidated like the 29 amino acid GAL and most biologically active peptides.

The gene encoding prepro-galanin (prepro-GAL) has been isolated from a number of species, including the human (Evans *et al.*, 1993), mouse (Kofler *et al.*, 1996) and rat (Vrontakis *et al.*, 1987; Kaplan *et al.*, 1988). In the human, the prepro-GAL gene has been localised to chromosome 11q13.3-13.5 (Evans *et al.*, 1993). While the length of the gene varies between species, ranging from 6.5kb (human) to 4.5kb (mouse), the organisation of the 6 exons and 5 introns within the gene appears to be conserved between species (Figure 1.4). Once the introns have been removed, the 6 exons are joined and form prepro-GAL and two untranslated transcript regions, with one at each end of prepro-GAL. Prepro-GAL is composed of a hydrophobic signal peptide (23 amino acids), a propeptide of 9 amino acids, the GAL coding region (29-30 amino acids) and a 59-60 amino acid GAL message-associated peptide (GMAP) (Rökæus & Brownstein, 1986; Iismaa & Shine, 1999) (Figure 1.4).

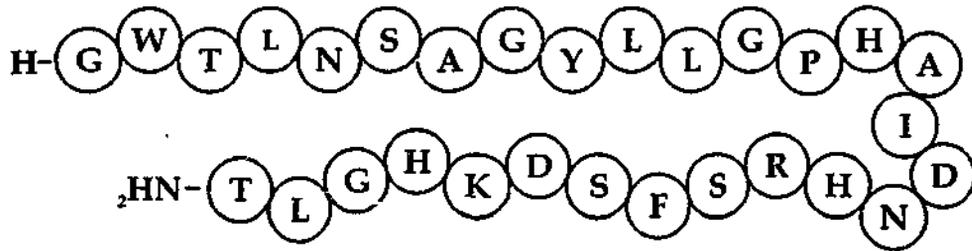
Expression of prepro-GAL mRNA has been detected in peripheral tissues such as the heart, gastrointestinal and genital tracts, liver, pancreas and adrenal gland (Kaplan *et al.*, 1988; Rökæus, 1994). Neurons containing prepro-GAL mRNA have been detected in the medial septum, BNST, amygdala (Ce and Me), hypothalamus (PVN, SON, DM, ARC, VMH and LHv), substantia nigra and claustrum. In the pons and medulla, prepro-GAL mRNA was detected in regions such as the PAG, LC, NTS, cerebellum and dorsal raphe nucleus (Gundlach *et al.*, 1990; Ryan & Gundlach, 1996; Iismaa & Shine, 1999).

**FIGURE 1.4**

Structure of prepro-GAL, the sequence of the GAL intermediate peptide prior to amidation and the final amino acid sequence of rat GAL.



AMIDATION



GALANIN

The distribution of the GAL peptide has been detected and quantified using a variety of immunohistochemical techniques. Neuronal perikarya containing GAL-ir have been found throughout all levels of the rat CNS in regions such as the BNST, medial septum, Ce, hippocampus, preoptic nuclei, ARC, DM, SON and magnocellular PVN. There were also cell bodies containing GAL-ir in the pons and medulla in regions including the dorsal raphe, LC, spinal trigeminal nucleus; caudal (Sp5C) and NTS (Melander *et al.*, 1986b; Skofitsch & Jacobowitz, 1986). GAL-ir has also been detected in fibre networks in many regions in the rat CNS, including the lateral septal nucleus, BNST, Ce, Me, preoptic areas, DM, PB, PAG, Sp5C and NTS (Melander *et al.*, 1986b; Skofitsch & Jacobowitz, 1986). In addition, GAL-ir fibres and cell bodies have been observed in parts of the rat spinal cord (Melander *et al.*, 1986b; Skofitsch & Jacobowitz, 1986).

A number of peripheral sites and organs in the rat also contain detectable levels of GAL-ir. These include the heart, gastrointestinal and genitourinary tracts, adrenal medulla and pancreas (Melander *et al.*, 1985; Bauer *et al.*, 1986; Pelto-Huikko, 1989; Hökfelt *et al.*, 1992; Xu *et al.*, 1995). Investigations of the distribution of GAL-ir throughout the body has not been limited to rats, with GAL-ir detected in many organs and regions in a number of animal species (Pelto-Huikko, 1989; Xu *et al.*, 1995). In the human, the presence of GAL-ir has been reported in regions such as the CNS and genitourinary tract (Bauer *et al.*, 1986; Gentleman *et al.*, 1989).

### 1.3.2.2 GAL receptors

Autoradiographic studies have used ligands such as [<sup>125</sup>I]-GAL to describe the distribution of GAL receptors in the CNS and periphery of a number of species (Köhler *et al.*, 1989; Moons *et al.*, 1991; Lundquist *et al.*, 1993). In the rat CNS, GAL receptors have a widespread distribution throughout the entire neuraxis. Previously published studies have reported the presence of [<sup>125</sup>I]-GAL binding sites in forebrain in regions such as the OB, amygdala, Pir, Ent, hippocampus, st, thalamus and various hypothalamic nuclei (Skofitsch *et al.*, 1986; Melander *et al.*, 1988). The midbrain, pons and medulla also contained regions where [<sup>125</sup>I]-GAL binding sites were detected such as the PAG, VTA, dorsal raphe, cuneiform nucleus (CnF), LC, Sp5C and NTS (Skofitsch *et al.*, 1986; Melander *et al.*, 1988). Peripherally, [<sup>125</sup>I]-GAL binding sites have been detected in the pancreas and gastric and jejunal smooth muscles of the rat (King *et al.*, 1989).

In recent years, three GAL receptors (GAL-R1, GAL-R2 and GAL-R3) have been cloned in the rat and human (see Table 1.1). All GAL receptors are members of the G protein-coupled superfamily that have 7 transmembrane domains. The relatively recent advances in the cloning of different GAL receptor subtypes have led to the synthesis of probes to investigate the distribution of the receptor mRNA for each subtype throughout the CNS and periphery (Table 1.1). Further characterisation of the effector systems of the three GAL receptor subtypes has also been reported. Presently, there are no specific antagonists that are able to discriminate between the different GAL receptor subtypes at the functional or autoradiographic level. As a consequence, there have been no reports of the subtype-specific distribution of GAL receptor protein in the CNS and periphery.

### 1.3.2.3 GAL and stress

Many studies have detailed changes in GAL neurochemistry following exposure to a variety of stressors such as social stress (visible burrow system) and cold temperatures (Malendowicz *et al.*, 1994; Holmes *et al.*, 1995). One of the earliest reports of a potential role for GAL in the stress response detailed the modulatory effect of GAL on the HPA axis of rats following exposure to ether (Hooi *et al.*, 1990). Infusion of GAL into the PVN of rats exposed to ether significantly attenuated the ether-induced increase in plasma ACTH concentration (Hooi *et al.*, 1990). Although this study demonstrates that GAL can inhibit the HPA axis during stress, contrasting results suggest that peripheral administration of GAL can stimulate the release of ACTH and aldosterone, but not corticosterone, following ether stress (Malendowicz *et al.*, 1994). Evidence for a possible stimulatory effect on the secretion of corticosteroids at the level of the adrenal cortex can account for the apparent discrepancy in the results of these two studies (Mazzocchi *et al.*, 1992; Malendowicz *et al.*, 1994). Malendowicz and colleagues also compared the effects of ether stress with a cold stress consisting of exposure to a temperature of 4°C for 20 min (Malendowicz *et al.*, 1994). In this study, the cold stress resulted in elevated plasma concentrations of ACTH, aldosterone and corticosterone of a magnitude about 65% of the increases elicited by ether stress. In contrast to the effects of ether, GAL (i.p.) did not induce any changes in plasma ACTH in cold-stressed rats. Further differences included a biphasic effect of GAL on cold-stress induced corticosterone release, with an initial potentiation followed by a suppression and an eventual return to normal stress levels (Malendowicz *et al.*, 1994). In summary, the modulatory effect of GAL on the HPA axis during stress depends on the type of

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**TABLE 1.1**

A brief description of the three cloned GAL receptor subtypes, GAL-R1, GAL-R2 and GAL-R3, which includes the distribution of the mRNA in the CNS and periphery of the rat, effector systems and species where the receptor has been cloned. For abbreviations, see page xvii.

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RECEPTOR	CLONED	mRNA DISTRIBUTION	EFFECTOR SYSTEM	REFERENCES
GAL-R1	RAT HUMAN	CNS – PVN, SON, ventral hippocampus, amygdala, thalamus, CTX, Sp5C, PB  Periphery – DRG, pancreatic islets, bladder, uterus	Inhibits cAMP accumulation via $G_i/G_o$ , increased $K^+$ currents via GIRK1, GIRK4	(Habert-Ortoli <i>et al.</i> , 1994; Parker <i>et al.</i> , 1995; Gustafson <i>et al.</i> , 1996; Landry <i>et al.</i> , 1998; Smith <i>et al.</i> , 1998; Yao <i>et al.</i> , 1998; Iismaa & Shine, 1999; O'Donnell <i>et al.</i> , 1999; Waters & Krause, 2000)
GAL-R2	RAT HUMAN	CNS – CTX, Pir, amygdala, hippocampus, DG, POA, mammillary nuclei, VMH, pPVN, VMH, ARC, dorsal hypothalamic area, LC, PAG, lat PB, XII  Periphery – genitourinary and gastrointestinal tracts, lung, kidney, DRG, pancreas	Stimulated phospholipase C via $G_{q/11}$ , increased intracellular $Ca^{2+}$ and inositol phosphate levels, inhibits cAMP accumulation via $G_i$	(Fathi <i>et al.</i> , 1997; Howard <i>et al.</i> , 1997; Smith <i>et al.</i> , 1997; Wang <i>et al.</i> , 1997; Ahmad <i>et al.</i> , 1998; Kolakowski <i>et al.</i> , 1998; Mitchell <i>et al.</i> , 1999; O'Donnell <i>et al.</i> , 1999; Waters & Krause, 2000)
GAL-R3	RAT HUMAN	CNS – hippocampus, hypothalamus, CTX, amygdala, cerebellum, medulla oblongata  Periphery – pituitary, lung, liver, kidney, spleen	Activated GIRK1 and GIRK4, possibly through $G_i/G_o$	(Kolakowski <i>et al.</i> , 1998; Smith <i>et al.</i> , 1998; Yao <i>et al.</i> , 1998; Waters & Krause, 2000)

stress, the magnitude of the stress and the anatomical location of the regulation (i.e. whether it is central or peripheral). As GAL can modulate the activity of the HPA axis at a number of different levels under basal conditions (Balment & al Barazanji, 1992; Malendowicz *et al.*, 1994), further research would provide clearer insights into the association between GAL and the response of the HPA axis to stress.

During exposure to stress, GAL has also been shown to modulate the response of the sympathoadrenal system. Thus, GAL administered peripherally (i.v.) at a dose of 1nmol/kg potentiated the increase in plasma adrenaline, but not NAdr, in response to a social stress stimulus (Ceresini *et al.*, 1998). This effect on catecholamine secretion may be mediated by a mechanism involving a currently unknown secondary organ/effector system, as i.v. GAL did not have any effect on catecholamine release during basal conditions (Ceresini *et al.*, 1998).

Physical stressors that alter the osmolarity or volume of the blood have been shown to alter central GAL neurochemistry in regions such as the PVN and SON (Meister *et al.*, 1990a; Yagita *et al.*, 1994). These alterations in GAL mRNA levels and GAL-ir occurred in brain regions that have been implicated in the regulation of the release of various hormones. GAL is colocalised with vasopressin in the rat PVN and SON (Melander *et al.*, 1986c; Skofitsch *et al.*, 1989), and GAL can modulate plasma levels of vasopressin and central vasopressin activity during exposure to stressors such as dehydration and i.p. administration of hypertonic saline (Kondo *et al.*, 1991; Landry *et al.*, 1995). The changes in prepro-GAL mRNA following osmotic stimuli are region-specific, as chronic oral ingestion of 2% saline did not alter expression of prepro-GAL mRNA in the LC (Foster *et al.*, 1992).

There has been further evidence indicating that activation of GAL-containing neurons within the LC depends on the type of stress. Exposure to a social stress stimulus induced a significant increase in the expression of prepro-GAL mRNA in the rat LC (Holmes *et al.*, 1995), while 3 consecutive days of 15 min swim stress did not induce any alterations in levels of the prepro-GAL transcript (Austin *et al.*, 1990). The data above indicate that GAL-containing neurons within the CNS are sensitive to perturbation by stress, and the activation or inhibition of these neurons depends on the type of stressor. Although there have been no comprehensive studies of the effects of any stress on GAL neurochemistry in the CNS, indirect evidence suggests that GAL may have a prominent modulatory role associated with neurotransmission during the stress response. For example, studies of neuronal activation following a variety of stressors described earlier (see section 1.1) have reported increased levels of Fos-ir and *c-fos* mRNA in numerous

central regions that also contain GAL-ir and/or prepro-GAL mRNA, such as the PVN and LC (Cullinan *et al.*, 1995). The effects of restraint, a psychological stressor that has been described earlier (section 1.2), has not been studied in relation to the central GAL system. Therefore, future studies that investigate changes in GAL neurochemistry should assist in clarification of the role of GAL in the neural response to stressors including restraint.

### 1.3.3 NEUROPEPTIDE Y

#### 1.3.3.1 *Synthesis and distribution of NPY*

NPY was purified from porcine brain extracts using a technique that identified peptides possessing C-terminal amide residues (Tatemoto *et al.*, 1982). NPY consists of 36 amino acids, and it was given its name because of the presence of the N-terminal Tyr and C-terminal Tyr amide residues (Tatemoto & Mutt, 1980). Since 1982, more than 50 peptides closely resembling NPY that have been isolated and characterised from numerous species (Larhammar, 1996). The NPY sequence has been identified in species such as the rat, rabbit, pig and human (Tatemoto, 1982; Corder *et al.*, 1988; O'Hare *et al.*, 1988; Jensen & Conlon, 1992). The sequence of NPY is highly conserved across species, with 22 of the 36 amino acids in identical positions in all species (Larhammar, 1996). Moreover, the rat and human NPY sequences are identical and differ from the porcine NPY sequence only at position 17.

NPY is formed following enzymatic cleavage of its precursor, prepro-NPY, and subsequent amidation of the carboxy terminal. The gene encoding prepro-NPY has been isolated in the rat (Larhammar *et al.*, 1987) and human (Minth *et al.*, 1986). In the human, the prepro-NPY gene has been localised to the pter-q22 region of chromosome 7 (Takeuchi *et al.*, 1986), in close proximity to the peptide YY (PYY) gene. In the rat and human, the prepro-NPY gene consists of 4 exons and 3 introns. The 4 exons encode (from 5' to 3') the 5' untranslated region, a 29 amino acid signal peptide, the 36 amino acid NPY peptide, a group of 3 amino acids essential for amidation, C-terminal flanking peptide of NPY (CPON; 30 residues) and the 3' untranslated sequence that contains the polyadenylation signal. During translation, the 5' and 3' untranslated regions are removed to form a prepro-peptide of 98 amino acids (Figure 1.5).

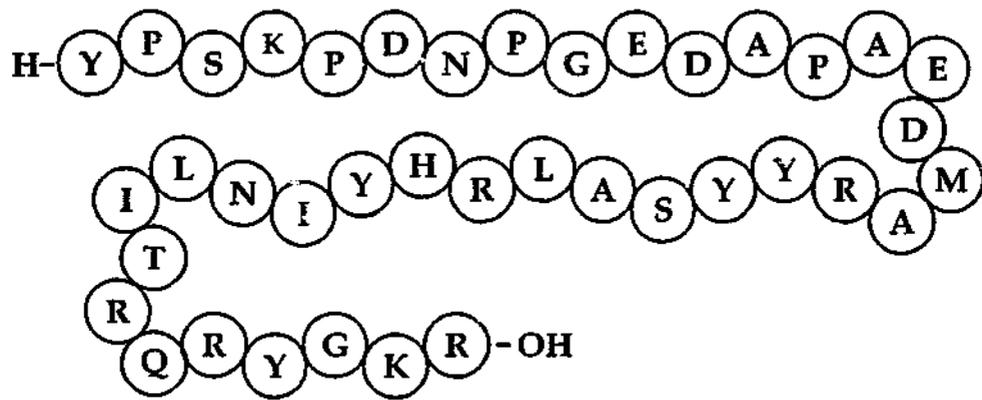
The prepro-NPY precursor has been detected throughout the CNS and in many peripheral tissues. In the CNS of the rat, the region containing the highest levels of the mRNA encoding

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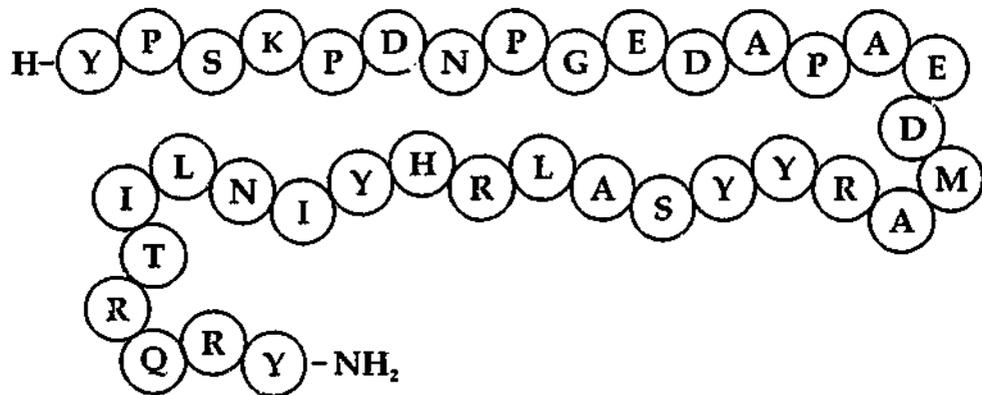
**FIGURE 1.5**

Structure of prepro-NPY, the sequence of the NPY intermediate peptide prior to amidation and the final amino acid sequence of NPY.

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AMIDATION



NPY

prepro-NPY is the hypothalamic ARC (Gehlert *et al.*, 1987; Morris, 1989; Houdeau & Boyer, 1994; McLean *et al.*, 1996). Forebrain regions containing a moderate to dense abundance of prepro-NPY mRNA include the cortex, hippocampus, DG, BM, Me, CPu, NAcc, reticular thalamus (Ret), DEn and olfactory tubercle. A lower density of prepro-NPY mRNA has been detected in other amygdaloid subnuclei including the Ce, the hypothalamus (PVN, Pe, DM, VMH, POA) and septum. In the midbrain, pons and medulla, regions containing prepro-NPY mRNA include the PAG, LC, CVLM, RVLM, NTS, sp5 and IOC (Gehlert *et al.*, 1987; Morris, 1989; Pieribone *et al.*, 1992; Houdeau & Boyer, 1994; McLean *et al.*, 1996).

Prior to the discovery of NPY in the porcine CNS, pancreatic peptide (PP) and PYY, two members of the pancreatic peptide (PP) family that share a structural similarity to NPY, were isolated from the avian pancreas and porcine intestine, respectively (Kimmel *et al.*, 1975; Tatemoto, 1982). These two peptides have been detected in peripheral tissues such as the pancreas and gastrointestinal tract and mediate a variety of effects (Bottcher *et al.*, 1993; Hazelwood, 1993). The peripheral distribution of NPY differs quite markedly from PP and PYY, with studies describing the presence of prepro-NPY mRNA in sympathetic (superior cervical and stellate) and sensory (nodose, petrosal and DRG) ganglia, heart, spleen, intestine, adrenal gland, lung and skeletal muscle (Larhammar *et al.*, 1987; Higuchi *et al.*, 1988; Czyzyk-Krzeska *et al.*, 1991; Pieribone *et al.*, 1992; Noguchi *et al.*, 1993; Hanze *et al.*, 1994).

When prepro-NPY enters the endoplasmic reticulum, the signal peptide is removed to generate the pro-hormone. Cleavage of the pro-hormone yields an intermediate NPY product of 38 residues and CPON that consists of 30 amino acids. Finally, a carboxypeptidase and peptidyl glycine alpha-amidating monooxygenase catalyse the cleaving and amidation of the intermediate product to generate the 36 amino acid amidated form of NPY (Figure 1.5).

Using immunohistochemical techniques, the distribution of NPY-ir has been described in detail throughout many different levels of the CNS and in a variety of peripheral tissues (Chronwall *et al.*, 1985; De Quidt & Emson, 1986). Moreover, there is a high correlation and similarity between the distributions of prepro-NPY mRNA and NPY-ir in the CNS and periphery. In the forebrain of rats, several reports have detected a high number of perikarya containing NPY-ir in the main and accessory OB, olfactory tubercle, cerebral cortex, lateral septum, BNST and ARC. Moderate populations of NPY-ir-positive neuronal cell bodies have been found in the Cing and olfactory cortex, DEn, CPu, NAcc, hippocampus, DG, ventrolateral geniculate nucleus, Me, Ce, BL, lateral amygdala and ventral pallidum. Regions such as the

PVN, medial and lateral POA contained a lower density of NPY-ir positive perikarya. These reports also detected dense networks of NPY-ir fibres in the Cing, DEn, claustrum, neocortex, hippocampus, DG, Me, Ce, medioventral NAcc, BNST, the majority of hypothalamic nuclei (PVN, ARC, POA, SON and suprachiasmatic area) and the PVA. NPY-ir fibres have also been detected in moderate to low densities in the cerebral cortex, various nuclei of the amygdala, septal complex, CPu, retrochiasmatic area and mammillary nuclei (Chronwall *et al.*, 1985; De Quidt & Emson, 1986).

In the mesencephalon and metencephalon, NPY-ir positive perikarya were found in high abundance in the PAG, dorsal raphe, LC, and dorsal tegmental nucleus. Other regions containing NPY-ir perikarya include the interpeduncular nucleus and inferior colliculus. Plexi of NPY-ir fibres have been detected in the PAG, dorsal raphe, PB, Barrington's nucleus and LC (Chronwall *et al.*, 1985; Yamazoe *et al.*, 1985; De Quidt & Emson, 1986). NPY-ir cell bodies and fibres have also been detected in the medulla oblongata and spinal cord. In the NTS, NPY-ir positive perikarya were found throughout the entire rostro-caudal extent of this nucleus, with a particularly high density of cells just rostral to AP (McLean *et al.*, 1999). Other nuclei where perikarya containing NPY-ir have been localised include the CVLM, RVLM, PrH, raphe obscurus and DMX. Axons and terminals containing NPY-ir have been detected in the NTS, spinal trigeminal nuclei, DMX, AP and Namb. Populations of lightly stained perikarya were found in the substantia gelatinosa at all levels of the spinal cord. In addition, laminae 1 and 2 of the cervical and thoracic spinal cord and a region dorsal to the central canal contained a moderate density of NPY-ir fibres (Everitt *et al.*, 1984; Chronwall *et al.*, 1985; Yamazoe *et al.*, 1985; De Quidt & Emson, 1986).

In the periphery, NPY-ir has been detected in the chromaffin and ganglion cells of the adrenal medulla (Varndell *et al.*, 1984; Oomori *et al.*, 1994), and these studies also found that NPY is colocalised with NAdr and tyrosine hydroxylase (TH) in the majority of these cells. Catecholaminergic neurons in regions such as the heart, spleen and skeletal muscle and surrounding the vasculature have been reported to contain NPY (Ekblad *et al.*, 1984; Lundberg *et al.*, 1985). NPY-ir has also been found in the nodose and trigeminal ganglia and genitourinary system of the rat (Tong *et al.*, 1996; Zhang *et al.*, 1996).

### 1.3.3.2 NPY receptors

All members of the PP family of peptides, including NPY, PP and PYY, are believed to act on the same receptors. These receptors, designated by a capital Y, belong to the superfamily of G-protein-coupled receptors containing seven helical transmembrane domains. As C-terminal fragments were reported to produce some, but not all, of the same physiological and functional effects of NPY, the existence of multiple subtypes of the Y receptor was postulated. Following the synthesis of a number of C-terminal fragments and NPY analogues, 6 NPY receptors have been characterised and designated  $Y_1 - Y_5$  and  $y_6$ . In Table 1.2, the distribution of the receptors for  $Y_1$ ,  $Y_2$ ,  $Y_4$  and  $Y_5$ , their second messenger systems and relative affinities for NPY and related peptides are described.  $Y_3$  and  $y_6$  have not been included, as the  $Y_3$  receptor has not been cloned and the  $y_6$  subtype has not been detected in any central or peripheral tissues in the rat at this stage (Burkhoff *et al.*, 1998).

### 1.3.3.3 NPY and stress

As described in earlier sections (section 1.1 and 1.2), the sympathetic nervous system has a significant role in the physiological and behavioural stress response. As NPY has been colocalised in catecholaminergic neurons in both the periphery and CNS (Lundberg *et al.*, 1982; Everitt *et al.*, 1984), it has the potential to modulate the stress-induced activation of these neurons. Supporting a role for NPY in the sympathetic component of the stress response was the report demonstrating that periods of high frequency stimulation (such as stress) result in the release of NPY from catecholaminergic nerves (Lundberg *et al.*, 1986). Furthermore, a significant amount of data have been published that detail changes in central and peripheral NPY neurochemistry following exposure to a variety of physical and psychological stressors.

Elevated plasma levels of NPY were recorded in rats exposed to acute stressors such as a novel environment, haemorrhage, footshock, handling, immobilisation and cold stress (Castagné *et al.*, 1987; Zukowska-Grojec *et al.*, 1988; Zukowska-Grojec & Vaz, 1988). While studies have reported changes in peripheral NPY neurochemistry following various stressors (e.g. Nankova *et al.*, 1996), numerous studies have detailed specific alterations in central prepro-NPY mRNA expression, NPY-ir or receptor density in the rat that were induced by stressful stimuli. Acute exposure to psychological stressors such as restraint and immobilisation results in significant

**TABLE 1.2**

A brief description of the Y receptor subtypes, including Y<sub>1</sub>, Y<sub>2</sub>, Y<sub>4</sub> and Y<sub>5</sub>. Information presented in the table covers the species where the receptor subtypes have been cloned, the distribution of the receptors in the CNS of the rat as reported by autoradiographic studies, effector systems and relative affinities for NPY, related peptides and fragments. The Y<sub>3</sub> and Y<sub>6</sub> subtypes have not been included as they are either not cloned at present (Y<sub>3</sub>) or undetected in the rat (Y<sub>6</sub>). For abbreviations, see page xvii.

RECEPTOR	CLONED	RECEPTOR DISTRIBUTION	EFFECTOR SYSTEM	LIGAND AFFINITY	REFERENCES
Y <sub>1</sub>	RAT HUMAN MOUSE DOG	CNS – anterior olfactory nuclei, claustrum, olfactory tubercle, CTX, DG, hippocampus, thalamus, PVN, VMH, Ce, BL, PAG, cerebellum, NTS, IOC	Inhibited cAMP accumulation and increased influx of Ca <sup>2+</sup> through G <sub>i</sub> /G <sub>o</sub> , but has also inhibited Ca <sup>2+</sup> currents in DG cells	HIGH - Full length NPY and PYY peptides  LOW – C-terminal fragments	(Westlind-Danielsson <i>et al.</i> , 1987; Eva <i>et al.</i> , 1990; Eva <i>et al.</i> , 1992; Larhammar <i>et al.</i> , 1992; McQuiston <i>et al.</i> , 1996; Nihlén <i>et al.</i> , 1997; Dumont & Quirion, 2000)
Y <sub>2</sub>	RAT HUMAN MOUSE GUINEA-PIG	CNS - OB, Pir, amygdala, hippocampus, st, lateral septum, LH <sub>y</sub> , ARC, medial POA, SN, VTA, AP, DR, NTS	Inhibited forskolin-stimulated cAMP accumulation, inhibited N-type Ca <sup>2+</sup> channels, increased/decreased Ca <sup>2+</sup> currents, G <sub>i/o</sub> and G <sub>s</sub>	HIGH – C terminal fragments, NPY, PYY	(Toth <i>et al.</i> , 1993; Gerald <i>et al.</i> , 1995; Dumont <i>et al.</i> , 1996; Nakamura <i>et al.</i> , 1996; Sharma <i>et al.</i> , 1998; St-Pierre <i>et al.</i> , 1998; Sun <i>et al.</i> , 1998; Dumont <i>et al.</i> , 2000)
Y <sub>4</sub>	RAT HUMAN MOUSE	CNS - OB, anterior olfactory nuclei, hippocampus, AP	Increased influx of Ca <sup>2+</sup> , inhibited accumulation of cAMP, activated GIRK1 through G <sub>i/o</sub>	HIGH – PP, PYY  MOD – NPY	(Bard <i>et al.</i> , 1995; Lundell <i>et al.</i> , 1995; Gregor <i>et al.</i> , 1996; Lundell <i>et al.</i> , 1996; Sun <i>et al.</i> , 1998; Dumont & Quirion, 2000)
Y <sub>5</sub>	RAT HUMAN MOUSE DOG	CNS – Anterior olfactory nucleus, olfactory tubercle, lateral septum, hippocampus, hypothalamus, amygdala, NTS, AP	Inhibited forskolin-stimulated cAMP synthesis	HIGH – NPY  MOD – PYY  LOW - PP	(Gerald <i>et al.</i> , 1996; Hu <i>et al.</i> , 1996; Borowsky <i>et al.</i> , 1998; Moser <i>et al.</i> , 2000)

reductions in prepro-NPY mRNA expression in neurons in regions such as the ARC, amygdala and neocortex (Thorsell *et al.*, 1998; Krukoff *et al.*, 1999), while no changes were observed in the LC, striatum and hypothalamus (Thorsell *et al.*, 1998; Makino *et al.*, 2000). NPY also appears to be involved in the central response to chronic psychological stress, as prepro-NPY mRNA levels were markedly different in the amygdala and ARC when compared to the acute response in the same region (Makino *et al.*, 1999; Thorsell *et al.*, 1999; Makino *et al.*, 2000). Furthermore, these changes in NPY gene expression are often associated with alterations in NPY peptide levels, with chronic immobilisation resulting in a reduced NPY content of the ARC (Pralong *et al.*, 1993).

Acute and chronic exposure to physical stressors such as salt loading, water and food deprivation and physical exercise have all been reported to increase the expression of prepro-NPY mRNA and NPY-ir in the rat hypothalamus, particularly in the PVN, ARC and SON (Larsen *et al.*, 1992a; Larsen *et al.*, 1992b; Lewis *et al.*, 1993; O'Shea & Gundlach, 1995). Hypotension induced by haemorrhage also increased the synthesis of prepro-NPY mRNA in medullary catecholaminergic nuclei such as the CVLM and RVLM (Chan & Sawchenko, 1998). In addition, drug-induced hypotension activated NPY-containing neurons in the NTS, CVLM and RVLM (McLean *et al.*, 1999).

## 1.4 HYPERTENSION

Disease states that result in an altered state of homeostasis can potentially contribute to aberrant and dysfunctional responses to acute and chronic stress. In one study, chronic ethanol consumption was shown to attenuate adaptation to chronic restraint (Haleem, 1996). Furthermore, there is a large body of data strongly suggesting that hypertensive subjects exposed to various stressors do not cope as well as subjects with normal BP. These studies have sometimes used hypertensive human subjects, together with patients considered to have a high risk of developing hypertension, with increased sensitivity to mental stress detected in the sympathoadrenal system, HPA axis and cardiovascular system in these hypertensive or borderline hypertensive patients (Kawabe *et al.*, 1994; Jern *et al.*, 1995; al'Absi *et al.*, 1998). An interesting study in Umbria in Italy also demonstrated how much influence the stress of everyday

life can have on BP levels (Timio *et al.*, 1997). Two groups of female subjects were selected and matched for age and BP – one consisted of residents from the region of Umbria, while the other included nuns from surrounding nunneries. Over a 30 year period, the nuns maintained a relatively stable BP. In contrast, the mean BP of the lay women increased steadily over the 3 decades, and this was also associated with a greater incidence of cardiovascular related diseases (Timio *et al.*, 1997). The authors attributed the BP differences between the two groups of women to varied lifestyle, in particular to the differences in stress experienced during the 30 year period.

The majority of studies, however, have used rodent models of hypertension in an attempt to study the basic neurochemical and physiological mechanisms of the stress response and how it differs in the hypertensive state. Alterations in the stress response between hypertensive and normotensive rats have been documented (see section 1.4.4), in addition to the large body of evidence describing a variety of neurochemical and phenotypic differences observed in hypertensive rat strains (sections 1.4.2 and 1.4.3).

### 1.4.1 MODELS OF HYPERTENSION

The first report of a hypertensive rat strain was published in 1958 by Smirk and Hall, where the New Zealand Genetically Hypertensive rat was generated through inbreeding of rats selected for an abnormally high resting BP. The discovery of another genetically hypertensive strain of rats was unintentional. During the course of an unrelated study by a research group in Japan, a male Wistar-derived rat was found to have a resting BP that was significantly greater than other rats of the same strain. The hypertensive offspring of this initial ancestor were selectively inbred, eventually leading to the generation of the SHR line (Okamoto & Aoki, 1963; DeVito *et al.*, 1981). However, SHR rats were distributed to other laboratories before the inbreeding program had produced more than 20 generations, consequently increasing the genetic variability between separate SHR colonies. In addition to this oversight, generation of a suitably inbred normotensive strain to provide an adequate control group for the SHR was not commenced until some time after the introduction of the SHR, with this control normotensive strain subsequently known as WKY.

In comparison to WKY rats that have a basal BP of 100 mmHg to 120 mmHg, resting BP in SHR starts to rise dramatically from 4 weeks of age until it reaches an elevated level anywhere

between 160mmHg and 240mmHg at 12 to 16 weeks of age (Iams *et al.*, 1979; Qualy & Westfall, 1988; Woo *et al.*, 1993). The elevation in BP occurs spontaneously in all SHR progeny (hence the name), and without any requirements for environmental influences. The origin of the polygenically inherited hypertensive trait in SHR is believed to be multifactorial, with research groups attempting to elucidate the genes responsible for producing a sustained elevation in BP. During the development of hypertension, peripheral sympathetic nerve activity is high, resulting in increased peripheral vascular resistance, elevated BP and eventual cardiovascular remodelling that contributes to maintenance of the hypertensive state in later life (Judy *et al.*, 1976; Judy *et al.*, 1979; Folkow, 1982). These haemodynamic changes were subsequently found to be similar to humans with genetic hypertension, leading many research groups to utilise the SHR strain as an animal model of essential hypertension (Yamori, 1977).

In recent times, the SHR strain has been proposed and validated as an animal model of attention deficit/hyperactivity disorder (AD/HD) due to the hyperactive phenotype (Sagvolden *et al.*, 1993; Sagvolden, 2000). Yamori and colleagues have developed the stroke-prone SHR through selected inbreeding of SHR that had died of stroke (Yamori *et al.*, 1974). The SHR-SP strain exhibits a higher MAP than SHR and the stroke-resistant SHR, and SHR-SP have a much shorter life span. In addition, selective breeding of SHR with WKY has resulted in the development of additional strains that facilitate research into particular aspects of the SHR phenotype. Firstly, Hendley and colleagues developed two strains of WKY where the hyperactive and hypertensive phenotype characteristic of SHR have been separated, so that a hypertensive strain with normal levels of activity and a normotensive hyperactive strain now exist (Hendley *et al.*, 1991). Lastly, the borderline hypertensive rat (BHR), also generated by breeding SHR and WKY, has a BP in the high normotensive range. This strain of rat, which represents an experimental model of hypertension, can develop hypertension following exposure to environmental stress or a high salt diet (Lawler *et al.*, 1981; Lawler *et al.*, 1987).

Additional experimental models of hypertension have also been developed, where surgery or treatment produces the elevated BP status. Models that fall into this category include the renal artery clip and deoxycorticosterone acetate (DOCA) salt models (Pinto *et al.*, 1998). Comparisons between experimental and hereditary models of hypertension can therefore provide valuable information, assisting in the classification of differences between hypertensive and normotensive rats as related to the hypertensive state or simple phenotypic variations (Sitsen & de Jong, 1983; Widz-Tyszkiewicz & Czlonkowski, 1991).

## 1.4.2 NEUROCHEMICAL DIFFERENCES IN THE SHR MODEL

Neurochemical alterations within the CNS are believed to be major contributors to both the development and maintenance of the hypertensive state in SHR. Many studies have reported changes in the concentration and activity of a variety of neurotransmitters/neuropeptides and associated changes in receptor density and receptor function in the CNS of SHR compared to WKY. Thus, despite the many peptides/transmitters that have been implicated in hypertension, the following section will only focus on neurochemical differences in the central opioid, GAL and NPY systems in SHR when compared with normotensive rat strains.

### 1.4.2.1 Opioids

The association between the central opioid system and cardiovascular regulation suggests that significantly different levels of opioid precursor mRNA, peptide concentration or receptor density may be detected in hypertensive disease states. To investigate the presence of these neurochemical differences, various facets of the opioid system have been compared in the CNS of hypertensive and normotensive rat strains. Using RNA extracted from grossly dissected brain regions, Hoegler and co-workers found that in SHR the hypothalamus, midbrain, cerebellum and spinal cord contained increased levels of prepro-ENK mRNA compared to WKY (Hoegler *et al.*, 1989). Additionally, a significantly reduced expression of prepro-ENK mRNA was detected in the combined pons and medulla brain region (Hoegler *et al.*, 1989). These results provide a target for a better anatomical investigation of regional prepro-ENK mRNA differences between hypertensive and normotensive strains using techniques such as ISHH. Using this technique, the expression of prepro-ENK mRNA was found to be significantly increased in neurons of the hypothalamus (ARC, DM, VMH medial POA), amygdala (Me, BM), LC and Pir and decreased in the NTS, CVLM and RVLM of SHR compared to normotensive WKY (Boone & McMillen, 1994b; Yin *et al.*, 1996b). Moreover in SHR, significantly reduced levels of proDYN mRNA have been detected in the hippocampus, DG, medial POA and NTS when compared to WKY rats (Wang *et al.*, 1994b; Yin *et al.*, 1997). Yin and colleagues also compared the levels of POMC mRNA in the ARC of both SHR and WKY and reported that the ARC of SHR contained a significantly higher expression of the POMC transcript (Yin *et al.*, 1997).

A number of studies have also detailed significantly different levels of peptide-ir in the CNS of SHR compared to WKY. Dynorphin-ir was significantly reduced in the hippocampus, hypothalamus (PVN) and Ce of SHR compared to WKY (Feuerstein *et al.*, 1983; Li *et al.*, 1989), while the CPu, substantia nigra and PAG of SHR had increased dynorphin A and dynorphin B concentrations (Feuerstein *et al.*, 1983; Tan-No *et al.*, 1997). In the CPu,  $\beta$ -endorphin-ir was found to be significantly lower in SHR compared to WKY levels, and Met-enkephalin-ir was elevated in the cortex and combined pons/medulla of SHR compared to normotensive controls (Bhargava *et al.*, 1988).

The changes in mRNA expression and peptide levels detailed in studies such as those above may induce or be a result of changes in opioid receptor density in the CNS of rats with elevated BP. The reduction in hippocampal dynorphin-ir previously observed in SHR appears to be paralleled by a significant reduction in  $\kappa$ -opioid receptors in the same region when compared to WKY (McConnaughey *et al.*, 1992). Other studies have detected an increased density of  $\mu$ - and  $\kappa$ -opioid receptors in the SHR hypothalamus (both), cortex ( $\kappa$ ) and midbrain ( $\mu$ ) compared to WKY (Bhargava & Das, 1986; Gulati & Bhargava, 1990). Furthermore, SHR rats appear to have a reduced  $\mu$ -opioid receptor and an increased  $\delta$ -opioid receptor density in the amygdala (Gulati & Bhargava, 1990; Bhargava & Rahmani, 1993). These studies were all performed using membrane preparations from selected brain regions, and there have been reports published that have used a standard autoradiography protocol with slide-mounted sections to provide a more detailed anatomical comparison of opioid receptor density in the CNS of normotensive and hypertensive rats. Using the  $\mu$ -selective ligand [ $^3$ H]-DAGO, Kujirai and colleagues (Kujirai *et al.*, 1991) revealed significantly elevated  $\mu$ -opioid receptor density in many regions including the CPu, Cing, VTA and DG. The study by Kujirai and co-workers also examined the density of  $\delta$ -opioid receptors using the  $\delta$ -selective ligand [ $^3$ H]-DPDPE and found an increased density of  $\delta$ -opioid receptors in regions such as the CPu and PAG (Kujirai *et al.*, 1991). In addition, various hypothalamic nuclei in SHR were found to contain a significantly increased density of  $\delta$ -opioid receptors, while the density of  $\mu$ -opioid receptors were significantly lower in the BL and NTS of SHR compared to WKY (Yin *et al.*, 1996a).

While changes in opioid neurochemistry in hypertensive rats may indicate that the opioid system is a primary influence on the altered hypertensive state in these strains, the changes may also contribute to other phenotypic differences between the strains. One such difference is an increased activity in SHR compared to WKY (Tsai & Lin, 1988), and significant alterations in

opioid receptor density and peptide levels in the CPU of SHR (Bhargava *et al.*, 1988; Kujirai *et al.*, 1991) may be associated with this behavioural trait. However, strong evidence of an association between the central opioid system and hypertension has been provided by functional studies. One study has shown that chronic s.c. naloxone treatment attenuated the development of hypertension in SHR (Quock *et al.*, 1984), suggesting that activation of the opioid system may contribute to the pathogenesis of hypertension. Furthermore, the non-selective opioid antagonist naltrexone lowers BP in SHR, but not WKY, when administered centrally (Levin *et al.*, 1986). Further studies have suggested that the hypertension and tachycardia observed following stimulation of central  $\mu$ -opioid receptors by i.c.v. morphiceptin in SHR may contribute to the hypertensive state, as the same concentration of morphiceptin administered i.c.v. resulted in hypotension and bradycardia in WKY (Widy-Tyszkiewicz & Czlonkowski, 1991). A similar pattern was also observed following i.c.v. U50-488H, with dose-dependent increases in BP and HR in SHR but the opposite cardiovascular effects in WKY. Adding further complexity to the results of the Widy-Tyszkiewicz study (Widy-Tyszkiewicz & Czlonkowski, 1991) was the observation that i.v. administration of U50-488H produced a similar hypotension and bradycardia in both WKY and SHR rats. However, the cardiovascular effects of U50-488H were not blocked by naloxone (i.v.) and as such may be mediated by a non-opioid receptor (Widy-Tyszkiewicz & Czlonkowski, 1991). In the hippocampus, direct microinjection of the  $\kappa$ -opioid agonist dynorphin A (1-8) resulted in a significantly larger hypotension and bradycardia in anaesthetised SHR compared to WKY (Wang & Ingenito, 1994a). In contrast, Sun and colleagues found that the  $\kappa$ -opioid receptor agonist U50-488H had no effect on BP or HR when injected into the dorsal hippocampus of anaesthetised WKY or SHR, with the difference in responses potentially attributed to the selectivity of the ligands (Sun *et al.*, 1996). Moreover, while  $\mu$ - and  $\delta$ -opioid receptor agonists did not alter BP or HR in WKY, the  $\mu$ -/ $\delta$ -opioid agonist DADLE elicited a depressor and bradycardic response in the dorsal hippocampus (Sun *et al.*, 1996). As an additional test, naloxone was microinjected into the dorsal hippocampus, PVN and RVLM of anaesthetised WKY and SHR. The results revealed that BP and HR was increased following injection into all three regions in WKY, but no effect was seen in SHR, providing further evidence for alterations in opioid neurochemistry that may contribute to the hypertensive phenotype of SHR (Sun *et al.*, 1996).

### 1.4.2.2 GAL

Studies that have measured GAL neurochemistry in the CNS of hypertensive rats have reported significant differences when compared to age-matched normotensive controls. Kunkler and colleagues assessed the GAL content and prepro-GAL mRNA expression of the LC and NTS in 4 week and 12 week old SHR and WKY rats (Kunkler *et al.*, 1994). In the 4 week old SHR group, significant increases were detected in GAL peptide levels in the LC and NTS and also in prepro-GAL mRNA expression in the LC. At 12 weeks, only GAL peptide levels in the NTS remained significantly elevated in the SHR compared to WKY. These results were taken to suggest that GAL-containing neurons within the NTS, and not the LC, may contribute to the maintenance of genetic hypertension. In the NTS, GAL can modulate cardiovascular reactivity as intra-NTS microinjection of GAL in anaesthetised rats suppressed the baroreceptor reflex response without altering basal HR and BP (Shih *et al.*, 1996).

Functional differences between hypertensive and normotensive rats related to GAL have been reported at the level of the medulla oblongata. GAL was found to dose-dependently inhibit the release of NAdr from slices of medulla oblongata dissected from both WKY and SHR rats (Tsuda *et al.*, 1992). A comparison of the magnitude of inhibition between strains found that the GAL-mediated inhibition of NAdr release in rat medulla slices was significantly attenuated in the SHR strain. Addition of pertussis toxin attenuated the GAL-mediated inhibition of NAdr release (Tsuda *et al.*, 1992), suggesting that either GAL-R1 or GAL-R3 receptor subtypes are mediating this cardiovascular activity of GAL. The  $\alpha_2$  adrenoceptor shares a similar signal transduction pathway to GAL-R1 and GAL-R3, which is through the pertussis toxin-sensitive G<sub>i</sub> protein (Jakobs, 1985). Therefore, the GAL-R2 receptor subtype is equally likely to be involved in the inhibition of NAdr release in the medulla if its actions are mediated through  $\alpha_2$  adrenoceptors or another receptor linked to a pertussis toxin-sensitive second messenger system. Investigation of the mechanism of NAdr release by GAL revealed that  $\alpha_2$  adrenoceptors could partially mediate this effect (Tsuda *et al.*, 1992). An earlier study reported significantly decreased inhibition of NAdr release by  $\alpha_2$  adrenoceptors in medullary slices of SHR compared to WKY (Tsuda *et al.*, 1990), which may be attributed to a decreased density of  $\alpha_2$  adrenoceptors in the medulla of hypertensive SHR (Yamada *et al.*, 1989a). A comparison of the density of GAL R1-R3 binding sites in the medulla awaits completion and should provide more insights into the role of GAL in the medulla of hypertensive SHR.

GAL can also inhibit the electrically-stimulated release of [ $^3\text{H}$ ]-dopamine in striatal neurons (Tsuda *et al.*, 1998). In contrast to the results of Tsuda and colleagues (Tsuda *et al.*, 1992), the inhibition of dopamine release by GAL was found to be significantly increased in SHR rats compared to the normotensive WKY. This may have a direct influence on dopamine release in the striatum, as a separate study reported a significantly reduced release of dopamine from an *in vitro* striatum preparation in SHR compared to WKY (Linthorst *et al.*, 1990). Therefore, GAL may contribute to the increased locomotor activity previously observed in this hypertensive strain (Kolloch *et al.*, 1980; Danysz *et al.*, 1983). The altered relationship between GAL and dopamine observed in SHR may be partially attributed to the non-significant 78% increase in GAL peptide concentration in the striatum compared to WKY (Bucinskaite *et al.*, 1995). Increased levels of GAL peptide were also detected in the hippocampus (+89%), occipital cortex (+60%) and pituitary (+34%) in SHR compared to WKY, although only the elevated GAL content in the hippocampus was significant (Bucinskaite *et al.*, 1995).

#### 1.4.2.3 NPY

Neurochemical comparisons between normotensive rats and SHR have demonstrated that numerous central nuclei contain significantly different levels of receptor density, NPY-ir and prepro-NPY mRNA. Studies using ISHH or radioimmunoassay have reported elevated prepro-NPY mRNA expression in the ARC (McLean *et al.*, 1996; Krukoff *et al.*, 1999) and attenuated expression of prepro-NPY mRNA in the cerebral cortex (Higuchi *et al.*, 1993) of SHR compared to normotensive controls. While similar levels of prepro-NPY mRNA expression were detected in regions such as the striatum, hypothalamus and pons/medulla (Higuchi *et al.*, 1993), this study analysed grossly dissected brain regions. These anatomically limited reports of prepro-NPY mRNA levels in SHR indicate that there is a need for further investigations of prepro-NPY mRNA expression throughout more regions of the CNS of SHR using a protocol that provides a greater degree of anatomical resolution.

The ARC, anterior hypothalamus, VMH, DM and striatum contained elevated levels of NPY-ir, while NPY-ir levels were attenuated in the lateral POA, cortex, LC and spinal cord of SHR compared to normotensive WKY (Maccarrone & Jarrott, 1985; Maccarrone *et al.*, 1986; Clark *et al.*, 1991). Regions such as the PVN and NTS were found to have similar levels of NPY-ir in

both strains of hypertensive and normotensive rats (Maccarrone & Jarrott, 1985; Maccarrone *et al.*, 1986).

Differences in NPY receptor density have also been detected. Chang and colleagues reported that the hippocampus and cortex of SHR contained a significantly increased density of [ $^{125}$ I]-NPY binding sites, with similar receptor levels found in the hypothalamus, midbrain, striatum and pons/medulla compared to WKY (Chang *et al.*, 1986). However, the use of grossly dissected and homogenised brain regions did not allow a detailed anatomical description of NPY receptor binding (Chang *et al.*, 1986). Moreover, Chang and colleagues were unable to discern significant differences in receptor density in medullary regions that were detected in later studies using *in vitro* receptor autoradiography. A significantly higher density of [ $^{125}$ I]-NPY binding sites was detected in the AP of SHR compared to WKY (Nakajima *et al.*, 1987), while Aguirre and co-workers detected a significant increase in the density of [ $^{125}$ I]-PYY binding sites in the NTS of SHR compared to WKY (Aguirre *et al.*, 1995). Further analysis of these results indicated that the elevated density of NPY receptors in the NTS was associated with an increased density of the Y<sub>2</sub> receptor subtype (Aguirre *et al.*, 1995). Interestingly, significant differences in NPY receptor density were not reported in the dorsal vagal complex (DVC) of WKY and SHR (McLean *et al.*, 1996). The DVC consists of the NTS, AP and dorsal motor nucleus of the vagus (DMX), and the use of [ $^{125}$ I]-BH-NPY did not enable delineation and quantification of NPY receptor density in these separate regions. However, examination of the emulsion-dipped medulla sections by light microscopy demonstrated that NPY binding sites were present in the commissural NTS and AP, but not in the DMX. Therefore, as Nakajima (Nakajima *et al.*, 1987) and Aguirre (Aguirre *et al.*, 1995) reported significant increases in NPY receptor density in the AP and NTS respectively, McLean and colleagues (McLean *et al.*, 1996) should have detected a similar increase in NPY receptor density in the DVC. The discrepancy in results may be attributed to the ligands and their differential selectivity that were used in all three studies. Presently, more detailed studies of NPY receptor density in the forebrain and pons of SHR are required. The continued development of ligands with increased selectivity for the different NPY receptor subtypes should provide researchers with adequate tools to enable a detailed comparison of NPY receptor density throughout all levels of the SHR CNS.

The reports detailed above describe significant alterations in NPY neurochemistry in SHR in regions implicated in cardiovascular control (NTS, AP) and the regulation of other autonomic functions. Particularly in the NTS, functional studies have provided further evidence of altered NPY activity in SHR in relation to the regulation of the cardiovascular system. While

microinjection of NPY into the NTS of SHR results in a hypotension and bradycardia that has previously been observed in normotensive rats, the potency of NPY to elicit these cardiovascular effects is significantly altered in SHR compared to WKY (Takesako *et al.*, 1994; Yang *et al.*, 1997). Differences in the functional relationship between NPY and NAdr have also been observed in the NTS, as well as the PVN (Woo *et al.*, 1993; Yang *et al.*, 1997). As NAdr can also exert potent cardiovascular changes within these regions, changes in the interaction between NPY and NAdr can have a marked influence on BP status.

In addition to the increased level of resting BP, differences in the SHR phenotype exist in the regulation of pain, feeding and the HPA axis (Hashimoto *et al.*, 1989a; Van den Buuse & de Jong, 1989; Pich *et al.*, 1993b). NPY has been implicated in many of these phenotypical differences, with studies demonstrating that central (i.c.v.) administration of NPY enhanced hypoalgesia and caused anxiolysis in SHR, effects that were not observed in WKY (Pich *et al.*, 1993a; Pich *et al.*, 1993b). Furthermore, i.c.v. NPY failed to induce feeding or sedation in SHR at concentrations that effectively produced these responses in WKY (Pich *et al.*, 1993a; Pich *et al.*, 1993b). Compared with normotensive rats, significant alterations in the functional effects and concentrations of NPY have been reported in peripheral tissues such as the cardiovascular system, genitourinary system, adrenal gland, platelets and pancreatic islets (Fujimoto *et al.*, 1993; Higuchi *et al.*, 1993; Zukowska-Grojec *et al.*, 1993; Chen & Han, 1995; Tong *et al.*, 1996).

### 1.4.3 FUNCTIONAL DIFFERENCES IN SHR

As described in some studies in the previous section, the neurochemical changes observed in SHR may not solely contribute to the hypertensive state. Closer study of the SHR strain has revealed numerous phenotypic differences when compared to WKY and other normotensive controls, such as behavioural hyperactivity, hypoalgesia, increased HPA axis activity and increased sensitivity and reactivity to environmental and stressful stimuli (Knardahl & Sagvolden, 1979; Hashimoto *et al.*, 1989a; Van den Buuse & de Jong, 1989; Pich *et al.*, 1993b).

Elevated activity in the HPA axis of young SHR has also been postulated to contribute to the development of hypertension. In young SHR, i.v. administration of CRF resulted in a markedly lower plasma ACTH concentration when compared to WKY (Hattori *et al.*, 1986; Hashimoto *et al.*, 1989a). This relationship was found to recover by 11 weeks of age, with no difference

observed between strains regarding the amount of ACTH released by exogenous CRF. In addition, young SHR were found to have an anterior pituitary that was larger than WKY, which correlated with a greater adeno-hypophysial content of ACTH (Hausler *et al.*, 1984). ACTH also produced a significantly elevated release of corticosterone in young SHR than in WKY (Sowers *et al.*, 1981). Plasma corticosterone and aldosterone levels were elevated in young SHR compared to normotensive rats (Sowers *et al.*, 1981; Hashimoto *et al.*, 1989a), while contradictory reports regarding the relative levels of plasma ACTH and corticosterone in adult SHR and WKY have also been published (DeVito *et al.*, 1981; McMurtry & Wexler, 1981; Wexler & McMurtry, 1982; Imaki *et al.*, 1998).

The level of nociception in SHR was compared with normotensive controls, and SHR were subsequently found to be hypoalgesic (Sitsen & de Jong, 1983). Further comparison of WKY with rats with experimental hypertension (i.e. renal artery clip and (DOCA)-salt treated rats) revealed that these alternative models of hypertension exhibited a level of nociception that was similar to WKY, indicating that hypoalgesia is a phenotypic difference characteristic of SHR and not related to hypertension. Moreover, the hypoalgesia in SHR can be eliminated by peripheral administration of naloxone and enhanced by central (i.c.v.) microinjection of NPY, implicating both of these neurochemical systems in the genesis of this trait (Sitsen & de Jong, 1983; Pich *et al.*, 1993b).

Behavioural assessments of SHR have revealed a number of differences following comparison with WKY. SHR are reported to be hyperactive compared to WKY, but hypoactive if compared with the Wistar and Sprague Dawley (SD) normotensive strains (Sagvolden *et al.*, 1993). It is therefore imperative that the normotensive strain is selected wisely and behavioural comparisons are discussed accordingly. In the open field test, SHR display higher ambulation and exploratory rearing activity, with a decrease in grooming and defecation as compared with WKY (Van den Buuse & de Jong, 1989; Sagvolden *et al.*, 1993). Moreover, differences in activity between SHR and WKY may be related to deficits in central dopaminergic neurochemistry as discussed by Van Den Buuse and colleagues (Van den Buuse & de Jong, 1989).

### 1.4.4 SHR AND STRESS

In addition to the phenotypic differences detailed in the above section, SHR have been reported to display a characteristically heightened response to various stressors. In particular, many studies have demonstrated that components of the sympathetic nervous system in SHR, including plasma catecholamines, BP and sympathetic nerve activity, were hyper-responsive to different stressors. The amplitude of air-jet- and footshock-induced changes in mean arterial pressure (MAP) and renal sympathetic nerve activity were significantly larger in SHR than WKY (Knardahl & Hendley, 1990; Li *et al.*, 1997; Zhang & Thoren, 1998). In addition, the cardiovascular system of SHR does not appear to habituate to psychological stressors as quickly as that of WKY, with up to 7 days of repeated exposure to restraint required for the tachycardic response of SHR to achieve the same level of adaptation observed in WKY after 1 session of restraint (McDougall *et al.*, 2000). The release of the catecholamines, NAdr and adrenaline, induced by stress was also increased in SHR relative to WKY (Kvetnansky *et al.*, 1979; Yamamoto *et al.*, 1987; Saiki *et al.*, 1997).

A recently published study demonstrated how stress can have detrimental effects on a genetically-sensitive cardiovascular system (Mansi & Drolet, 1997). BHR rats were selectively bred from SHR and WKY rats and have a level of MAP in the high normotensive range. However, exposure of these rats to repeated stress produced a hypertension that may last for up to 3 months after cessation of the stress. This transient resetting of resting BP was associated with an increase in sympathetic tone and a hyper-reactive sympathetic response to stress (Mansi & Drolet, 1997). The BHR strain may therefore be a useful model for stress-induced hypertension that is particularly relevant to human studies.

Considering the role of the HPA axis in the stress response and the presence of strain-related differences in the activity of the HPA axis, the neuroendocrine limb of the stress response was the focus of early studies of the effects of stress on the SHR strain. These studies described conflicting changes in plasma corticosterone and aldosterone in response to stressors such as ether and immobilisation, with both larger and attenuated stress-induced elevations of plasma corticosterone and aldosterone when compared to normotensive SD and WKY rats (McMurtry & Wexler, 1981; Sowers *et al.*, 1981; Gómez *et al.*, 1996). The discrepancy in results may relate to a number of differences in the protocol, from the choice of normotensive control rats (SD or WKY), to the magnitude of the stressor (restraint or immobilisation). Furthermore, the age of

the SHR used in the study appears to be a major influence on the HPA axis response to stress, which is not surprising given the presence of many variations in HPA axis activity in young SHR that were described earlier (see section 1.4.3). A study by Hausler and colleagues demonstrated that younger SHR have a heightened responsiveness to ether stress than normotensive rats of the same age, while adult SHR, WKY and Wistar rats exhibit a similar corticosterone and ACTH response to the same stressor (Hausler *et al.*, 1983). In addition, some studies have reported an increased HPA axis response to stress in SHR (Imaki *et al.*, 1998). Additional hormonal systems in SHR also show an exaggerated response to stress, with larger stress-induced increases in plasma prolactin, glucose and renin observed in SHR compared to WKY (McMurtry & Wexler, 1981; Porter, 1990; Armario *et al.*, 1995).

The strain-related alterations in the neuroendocrine, sympathetic and physiological stress response are also paralleled by differences in the central stress response. Studies comparing the expression of Fos protein or *c-fos* mRNA in selected brain regions have demonstrated that stress does not activate the same central regions to a similar degree in the SHR and WKY strains. Regions such as the hypothalamus (PVN, VMH), LC, RVLM and NTS were identified as responding differently to an air-puff stimulus (Imaki *et al.*, 1998; Palmer & Printz, 1999). These regions, in particular the RVLM and NTS, are components of the central sympathetic neural network that regulates sympathetic outflow and autonomic function. In addition, the PVN is an essential region that governs the activity of many neuroendocrine systems. Thus, these data provide exciting targets for further research into the altered stress response in SHR.

## 1.5 AIMS AND OBJECTIVES

The present thesis aims to investigate the neurochemical response to acute and chronic restraint stress. As described in section 1.2, many studies have identified physiological changes associated with exposure to this particular neurogenic stressor. Research groups have also investigated the central changes occurring as a result of exposure to restraint using a variety of approaches. Some have measured restraint-induced alterations in neuronal activation using immunohistochemistry with antibodies targetted at Fos or other immediate early genes. Others have attempted to identify the phenotype of neurons involved in the central stress response.

However, restraint-induced changes in neurotransmitter and neuromodulator concentrations and receptor density have not been thoroughly investigated.

Of particular interest are the peptides belonging to the opioid family, enkephalin and dynorphin, and the peptides GAL and NPY. These peptides, together with their respective receptors, have been detected throughout all levels of the CNS. All of them are present in many of the major central autonomic control centres, including the PVN, LC, ventrolateral medulla and NTS. In light of these observations, these particular regions of the CNS will be the focus of the first 2 results chapters.

The opioids, GAL and NPY are implicated in the central regulation of various processes, including cardiovascular control, feeding regulation, neuroendocrine control and nociception (Heilig & Widerlöv, 1995; Iismaa & Shine, 1999; Vaccarino *et al.*, 1999). More importantly, studies have also demonstrated that stress can alter the central neurochemistry of these peptides. More specifically, psychological and physical stressors alter the expression of genes encoding their precursors, the concentration of the peptides in discrete brain regions, the density of their receptors and their functional relationship with other neurotransmitters (see sections 1.3.1.4, 1.3.2.3 and 1.3.3.3). However, the tools available at the time of the experiments have restricted some of these studies, with limitations on the selectivity and specificity of ligands and anatomical resolution of the technique. Through the utilisation of better protocols and experimental tools, the present thesis therefore aims to extend these studies and contribute to the ever-growing knowledge base that describes the sensitivity of these peptide systems to a psychological stressor.

The opioids, GAL and NPY can modulate both sympathetic outflow and associated cardiovascular parameters, and in addition, the neurochemistry of all three peptide systems have been reportedly altered in hypertensive rat strains (section 1.4.2). Studies have used a variety of approaches to compare the function of specific CNS regions in hypertensive rats. In regards to the opioid, GAL and NPY systems, some investigations have measured and compared changes in cardiovascular parameters elicited by particular agonists and antagonists in normotensive and hypertensive strains (e.g. Sun *et al.*, 1996; Yang *et al.*, 1997). Other studies have preferred to concentrate on a direct comparison of gene expression, peptide levels or receptor density in the CNS of normotensive and hypertensive rats (Bhargava *et al.*, 1988; McLean *et al.*, 1996). However, the majority of the neurochemical comparisons of the opioid, GAL and NPY systems in normotensive and hypertensive strains have been anatomically restricted to only a few central

regions or used non-selective ligands or techniques. There is therefore a requirement for detailed investigations of the CNS of normotensive and hypertensive strains, with a particular focus on the opioid peptides, GAL and NPY, using improved protocols and techniques.

The present thesis will use two different approaches to investigate the central neurochemistry in rats belonging to the hypertensive SHR strain and its progenitor normotensive strain, WKY. Firstly, differences in the basal level of gene expression of selected peptide precursors will be quantified using slide-mounted brain slices subjected to a standard ISHH protocol. Secondly, *in vitro* autoradiography with a variety of radioligands, also using slide-mounted brain sections, will be utilised to quantify strain-related differences in the density of opioid ( $\mu$ ,  $\delta$  and  $\kappa$ ) and GAL receptors. Using the combination of these two techniques will provide an anatomically detailed assessment of the opioid, GAL and NPY systems in the CNS of both normotensive and hypertensive strains that will compliment many of the earlier studies of the same peptide transmitters/modulators.

The differences in opioid, GAL and NPY neurochemistry that were observed in previous studies (and potentially in the present thesis) between SHR and normotensive controls may directly contribute to the pathogenesis and maintenance of the hypertensive state, but they may also contribute to some of the other phenotypic differences between these rat strains. These include hypoalgesia, hyperactivity and more importantly, an altered stress response. Thus, it is likely that a comparison of the neural response to restraint stress, where gene expression or receptor density is quantified for the opioid, GAL or NPY systems in the CNS of both normotensive and hypertensive rats may reveal significant variations between strains. As a consequence, the present thesis will also compare the neurochemical response to both acute and chronic restraint stress between WKY and SHR rats. Moreover, the present thesis will also attempt to identify whether basal alterations in gene expression or receptor density contribute to and/or influence the sensitivity of central neurons to acute and chronic restraint stress.

To compliment these investigations, the final results chapter will determine how the opioid system is involved in the neural response to acute restraint stress. The restraint-induced activation of central neurons will be quantified using immunohistochemistry in conjunction with an antibody directed at Fos protein. Fos is a commonly used marker of neuronal activation. One of the advantages of Fos immunohistochemistry is that the basal expression level of Fos is very close to the detection limits of the technique, such that any stimulus that activates a neuron and subsequently promotes Fos synthesis can be easily detected throughout the CNS.

Two groups of rats will be exposed to an acute 60 min session of restraint stress and subsequently processed for Fos immunoreactivity. The first group of rats will receive an i.c.v. microinjection of naloxone to produce a non-selective blockade of central opioid receptors immediately preceding the restraint session, while the second control group of rats will be injected with saline (i.c.v.). Comparison of the distribution and number of Fos-positive cells in selected regions of the CNS of both groups of rats will provide an indication of how opioid peptides modulate neurotransmission during exposure to a psychological stressor. Furthermore, the anatomical location of any differences may provide some insight into the physiological consequences of opioid receptor blockade during stress.

Therefore, the experiments undertaken in this thesis will examine how specific neuropeptides are involved in the neural response to both acute and chronic restraint stress. The thesis will also attempt to compare the sensitivity of three different neuromodulator systems (opioid, GAL and NPY) to a psychological stressor between WKY and SHR. Thus, a greater understanding of the central stress response, in particular the involvement of different neuropeptides and the effect of altered cardiovascular homeostasis on the stress response will be gained from the completion of this thesis.

# GENERAL METHODS



Method  
meth'əd.

Noun - the manner of performing any act or operation; a procedure or technique. A way of doing something, especially in accordance with a defined protocol.

## **CHAPTER 2**

### **GENERAL METHODS**

#### **2.1 ETHICS**

The experiments conducted as part of this thesis were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

#### **2.2 ANIMALS**

##### **2.2.1 PILOT STUDY TO VERIFY STRESS PARADIGM**

Male SHR rats (n=9; 40-44 weeks; 360-480g) were obtained from the Biological Research Laboratory, Austin Hospital, Heidelberg, Melbourne. Prior to experimentation, rats were chronically implanted with radiotelemetric probes that had been surgically inserted into the abdominal aorta at 14-16 weeks of age by Drs Widdop and Paull according to a published protocol (McDougall *et al.*, 2000). These radiotelemetric probes enabled continuous recording of BP and HR, thus providing accurate measurements of restraint-induced disturbances to the cardiovascular system. In addition, these rats had previously received an infusion of an angiotensin II AT<sub>1</sub> receptor antagonist for 4 weeks. At the time of drug infusion, the BP of these rats had dropped from 140 mmHg to 100 mmHg, and in the 8-16 week period after the drug infusion paradigm had been completed, the BP of these rats had returned to 140 mmHg. All rats were individually housed in a temperature- (22 ± 1 °C) and light-controlled room (lights on 07:00h) and were provided with water and standard laboratory rat chow (Clarke-King ARM) *ad libitum*.

## 2.2.2 IN SITU HYBRIDISATION HISTOCHEMISTRY (ISHH) AND AUTORADIOGRAPHY

Age-matched (16-18 weeks) male WKY (300-360g) and SHR (300-350g) rats were obtained from the Biological Research Laboratory, Austin Hospital, Heidelberg, Melbourne. All rats used in these experiments were housed in groups of 3 in a temperature-controlled room ( $22\pm 1^\circ\text{C}$ ) that was illuminated on a 12:12 hour light/dark cycle (lights on at 07:00h). Rats were provided with standard laboratory rat chow and water *ad libitum*.

## 2.2.3 IMMUNOHISTOCHEMISTRY

Male WKY rats (360-405g) were obtained from the Biological Research Laboratory, Austin Hospital, Heidelberg, Melbourne. Prior to stereotaxic surgery, WKY rats were housed in groups of 3 in a temperature-controlled room ( $22\pm 1^\circ\text{C}$ ) that was illuminated on a 12:12 hour light/dark cycle (lights on at 07:00h). After completion of the surgery, WKY rats were allowed to recover for at least 7 days where they were housed individually in the laboratory with standard rat chow and water *ad libitum*.

## 2.3 RESTRAINT STRESS PARADIGM

A restraint session started with a minimum 30 min equilibration period in the laboratory to allow the rats to acclimatise to the new surroundings. At the end of this period, each rat was placed in a plexiglass tube (6 cm diameter and 22 cm length; Plas Labs, Lansing, MI.) that had holes drilled in the end and sides to allow constant access to fresh air. Rats were prevented from turning around by an adjustable plastic plug that was secured behind the rat once it was positioned in the tube. Rats remained in the plexiglass tube for a period of 60 min, and food and water were not provided during this time.

## 2.4 PILOT STUDY - TELEMETRY AND RESTRAINT

### 2.4.1 METHODS

Rats were divided into 3 groups, with the first group exposed to restraint between 9:30h and 11:30h, the second exposed to restraint between 11:30h and 1400h and the third group exposed to the restraint paradigm between 13:00 and 16:00h. On the day of the experiment, HR and BP were recorded for 30 min prior to weighing. Immediately after weighing, rats were transferred to the restraint tube and returned to the home cage to resume telemetric recording of HR and BP. After the 60 min restraint period, rats were removed from the tube and returned to their home cage to record HR and BP for a further 30 min. Restraint tubes were also removed from the home cage once the rat had been released. Rats were subjected to this restraint protocol for 5 consecutive days, and during each recording period, general behavioural observations were recorded.

Note that after completion of the restraint paradigm, the rats were not used for any further experiments in this thesis.

### 2.4.2 RESULTS

During the restraint period, rats exhibited a state of general awareness, with piloerection, defaecation, urination and increased respiration observed in all rats exposed to the restraint stimulus. Some, but not all, rats vocalised during the restraint period. Upon release from the tube, the activity of the rats increased as they explored their home cage and eventually began to subside over time.

MAP was calculated according to the formula:

$$[ \frac{1}{3} ( \text{SYSTOLIC PRESSURE} - \text{DIASTOLIC PRESSURE} ) ] + \text{DIASTOLIC PRESSURE}$$

MAP and HR values were collected for each min of the 2 hour recording period, grouped into bins of 3, averaged and expressed as change in either MAP or HR. As shown in Figure 2.1, acute exposure to restraint produced a maximal tachycardic increase of ~200bpm in each of the 3 groups. Moreover, this maximal increase in HR was reproducible after 3 and 5 exposures to the same restraint paradigm. There were, however, differences between the morning, midday and afternoon groups in terms of HR adaptation to restraint during the 60 min period. The morning group (Figure 2.1A) appeared to adapt much slower to the restraint paradigm, with the temporal response to the 60 min restraint period on the first day very similar to that on the third and fifth day. In contrast, the midday (Figure 2.1C) and afternoon groups (Figure 2.1E) exhibited a more apparent difference between the HR response to the first restraint period and the third and fifth day of restraint. Furthermore, the average HR of the afternoon group almost reached resting levels during the restraint period on days 3 and 5. In all groups, release from the tube triggered another period of tachycardia that was comparable to the increase in HR observed when the rats were first restrained.

MAP responses to the restraint protocol were much more variable than HR. In all groups, 1 session of restraint produced the smallest pressor response, with 3 and 5 sessions eliciting a slightly larger increase in blood pressure. While there were differences between the morning group (Figure 2.1B) and the midday/afternoon groups (Figure 2.1D and 2.1F) in terms of the temporal MAP response to the first session of restraint, MAP responses induced by subsequent exposure to the restraint paradigm on the third and fifth day were similar between rat groups.

### 2.4.3 CONCLUSION

The results of this preliminary study suggest that rats exposed to a stressor (restraint) in the morning do not cope as well as rats exposed to exactly the same protocol in the afternoon. This variation in the stress response can be correlated with diurnal fluctuations in the HPA axis (Fuchs *et al.*, 1996). In the morning, plasma CRF levels are almost at their highest level, while plasma corticosterone concentrations are at their nadir. Moreover, central [<sup>125</sup>I]-CRF binding site density is highest between 9:00h and 13:00h in a number of brain regions, including the PVN, amygdala and LC (Fuchs *et al.*, 1996).

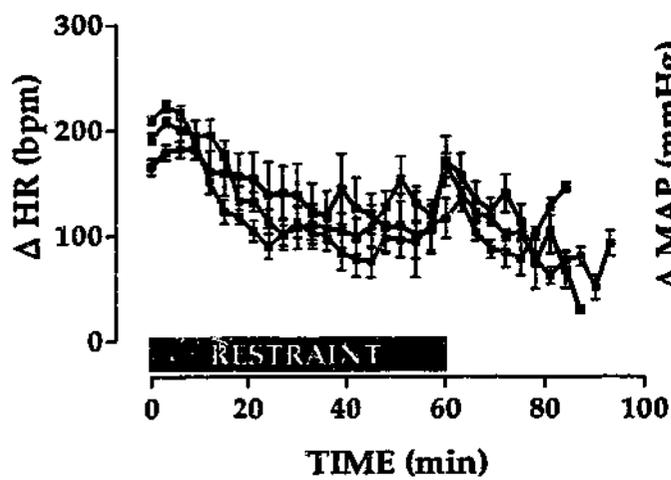
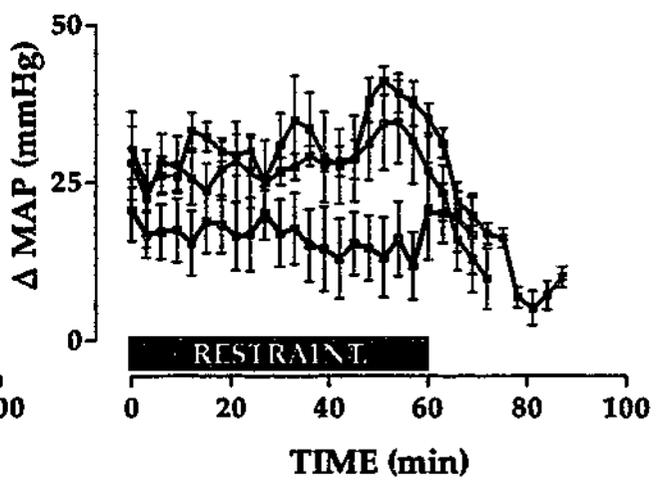
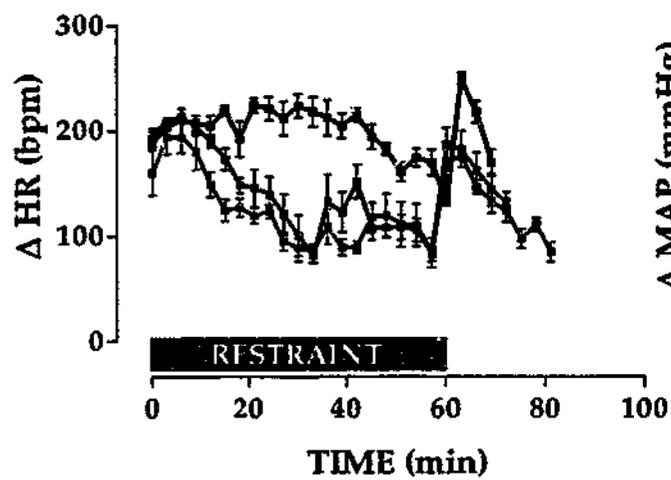
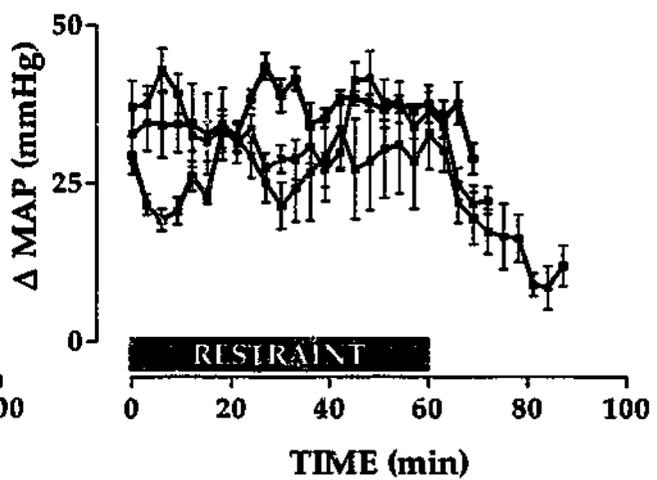
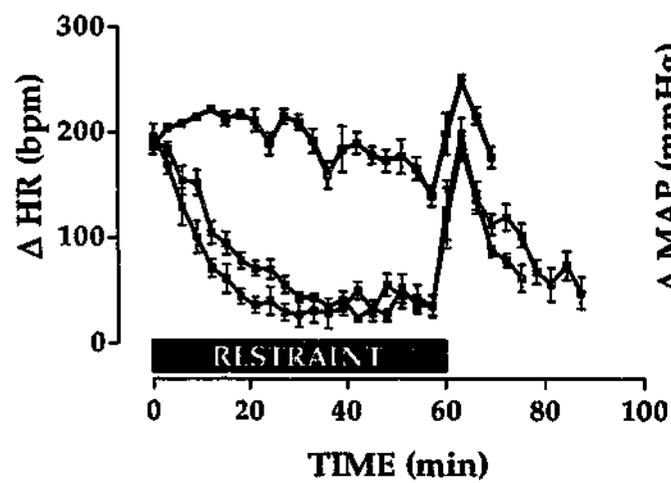
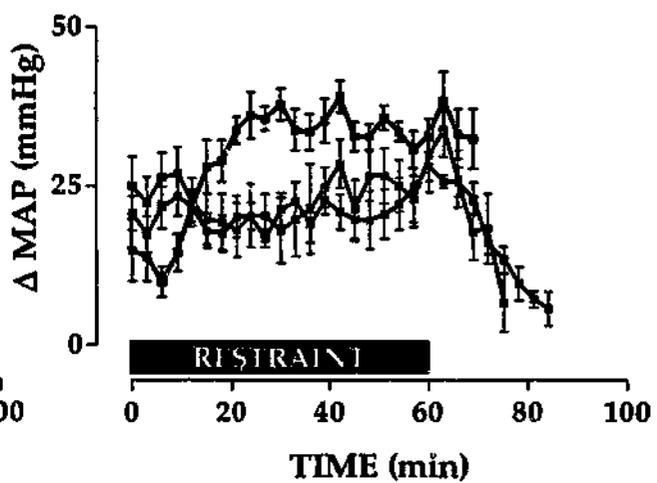
Therefore, as a consequence of this preliminary study, future studies using the restraint protocol will subject all rats to restraint stress between 10:00h and 12:00h. In addition, when

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**FIGURE 2.1**

Results of a preliminary study of the cardiovascular response to a 5 day restraint stress paradigm in aged SHR. Rats ( $n=9$ ) were divided into 3 groups (3 rats in each group), with group 1 (panels A and B) exposed to restraint stress in the morning, group 2 exposed to restraint at midday (panels C and D) and group 3 exposed to restraint in the afternoon (panels E and F). The restraint paradigm consisted of 60 min of restraint presented at a similar time each day for 5 consecutive days, and all rats had chronically-implanted radiotelemetric probes to enable continuous measurement of heart rate (HR) and mean arterial pressure (MAP). Change in HR (bpm; panels A, C and E) and MAP (mmHg; panels B, D and F) relative to resting levels are represented as mean  $\pm$  S.E.M. and are shown for day 1 (black), 3 (green) and 5 (red) of the restraint paradigm.

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**A****B****C****D****E****F**

rats are exposed to more than one period of restraint stress (as in chapters 3 and 4), each rat will be restrained at the same time each day to minimise the effects of diurnal variations on the stress response and subsequent results.

## 2.5 PREPARATION OF BRAIN SECTIONS FOR ISHH AND AUTORADIOGRAPHY

At the completion of the restraint paradigm, rats were decapitated using a standard guillotine. The brain was then dissected and removed, frozen over liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until required. On the day of sectioning, brains were removed from  $-80^{\circ}\text{C}$  storage and allowed to equilibrate to the temperature of the cryostat ( $-16^{\circ}\text{C}$ ). Brains were mounted onto chucks using Tissue-Tek® O.C.T. Coronal brain sections ( $14\mu\text{m}$ ) were cut on a cryostat (Cryocut 1800, Leica) at  $-16^{\circ}\text{C}$  at the following levels: forebrain at the level of the PVN/amygdala (bregma  $-1.80$  mm to  $-3.00$  mm), pons at the level of the LC (bregma  $-10.50$  mm to  $-9.16$  mm) and medulla oblongata at the level of the NTS (bregma  $-14.60$  mm to  $-12.80$  mm) (Paxinos & Watson, 1986). Sections were thaw-mounted onto glass microscope slides that had been coated in either poly-L-lysine (ISHH) or gel-chrome alum (autoradiography).

### 2.5.1 PREPARATION OF POLY-L-LYSINE COATED SLIDES

Poly-L-lysine was added to sterile diethyl pyrocarbonate (DEPC)-treated distilled water (distilled water obtained from the MilliQ filtering system containing 0.1% DEPC that had been autoclaved) to give a final poly-L-lysine concentration of  $5\mu\text{g/ml}$ . Glass microscope slides that had been baked at  $180^{\circ}\text{C}$  for at least 5 hours were then dipped in the poly-L-lysine solution and allowed to dry.

## 2.5.2 PREPARATION OF GEL-CHROME ALUM COATED SLIDES

The gel-chrome alum subbing solution was prepared using a standard protocol. Distilled water (500ml) was heated to between 40°C and 50°C, 2.5g of gelatin was added and stirred until completely dissolved. The gelatin solution was cooled to less than 30°C, 0.25g of chrome alum (chromium (III) potassium sulfate) was added and the solution was stirred until the chrome alum had dissolved. The subsequent solution was then filtered and was ready for use. Glass microscope slides were loaded into glass racks, dipped in distilled water, dipped twice in the gel-chrome alum subbing solution and dried overnight in an oven at 40°C.

## 2.6 ISHH

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### 2.6.1 FIXATION AND DELIPIDATION

On the day of fixation, the slide-mounted brain sections were removed from storage and allowed to warm to room temperature. Slides were placed in a glass rack and submerged in ice-cold 4% depolymerised paraformaldehyde (PFA; pH 7.0) for 5 min, transferred to 1 X phosphate-buffered saline (PBS) at room temperature for 3 min, and dehydrated through serial ethanol dilutions at room temperature (70, 95 and 100% for 2 min each). Sections were then delipidated in chloroform (20 min), rinsed in 100% ethanol for 2 min and stored in 95% ethanol at 0-4°C until further use (Table 2.1).

TABLE 2.1

SOLUTION	TIME (min)
4% PFA	5
1 X PBS	3
70% EtOH	2
95% EtOH	2
100% EtOH	2
CHLOROFORM	20
100% EtOH	2
95% EtOH	Storage

Protocol for fixation

### 2.6.2 ACETYLATION

Brain slices were acetylated prior to hybridisation, as preliminary experiments indicated that non-specific hybridisation was reduced using these conditions. Specifically, slides were transferred from 95% ethanol storage into a glass rack in 1 X PBS for 2 min. They were then placed in 0.1M triethanolamine (TEA)/0.9% saline buffer (pH 8.0) containing 0.25% acetic anhydride (AcAnh) for 10 min. Following acetylation, slides were rinsed in 70% ethanol (2 min), dehydrated in serial ethanol dilutions (95, 100%; 1 min), delipidated in chloroform for 5 min, rinsed in serial ethanol dilutions (100, 95%) for 1 min and dried at room temperature (Table 2.2).

TABLE 2.2

SOLUTION	TIME (min)
1 X PBS	2
0.1M TEA/ AcAnh	10
70% EtOH	2
95% EtOH	1
100% EtOH	1
CHLOROFORM	5
100% EtOH	1
95% EtOH	1

Protocol for acetylation

### 2.6.3 OLIGONUCLEOTIDE PROBE LABELLING

A number of different oligonucleotide probes were used in conjunction with this ISHH procedure, and the specific details of each probe are described in the appropriate methods section in Chapter 3. In each case, either a single probe (prepro-ENK) or a combination of probes (proDYN, prepro-GAL and prepro-NPY) was used. A working stock of 0.3 pmol/ $\mu$ l solution was prepared that contained either a single probe or a combination consisting of equal concentrations of each probe. Aliquots (5 $\mu$ l) were 3'-end labeled with [ $\alpha$ -<sup>33</sup>P]dATP (2000 Ci/mmol) using terminal deoxynucleotidyl transferase and cobalt chloride (1.4mM) to a specific activity of 1.0-2.5 X 10<sup>6</sup> DPM/ $\mu$ l. The labeled probe(s) was then applied to a Sephadex G25 column and spun at 2000 rpm (Clements GS200; Biotronics, Australia) for 1 min to separate any unincorporated nucleotides from the probe(s).

### 2.6.4 HYBRIDISATION PROCEDURE

The labeled antisense oligonucleotide probe(s) were diluted in a minimalist hybridisation buffer containing 50% de-ionised formamide, 10% dextran sulfate and 4 X saline sodium citrate (SSC = 0.6M NaCl, 0.06M sodium citrate, pH 7.0) and applied to adjacent sections of rat brain (1pg/ $\mu$ l, 100 $\mu$ l). Segments of Parafilm® were apposed to the slides to assist in covering the sections evenly with the hybridisation solution. The slides were then loaded into plastic petri dishes that contained tissue paper soaked in 50% de-ionised formamide containing 4 X SSC to provide a humidified atmosphere. The petri dishes were sealed with Parafilm® and placed overnight in an oven that had been preheated to 42°C.

**TABLE 2.3**

SOLUTION	TIME
1 X SSC	30 sec
1 X SSC (55°C)	60 min
1 X SSC	30 sec
0.1 X SSC	30 sec
70% EtOH	30 sec
95% EtOH	30 sec
95% EtOH	1 min

Protocol for washing of slides after hybridisation

On the following day, slides were removed from the petri dishes and the Parafilm® segments were carefully separated from the slides. Slides were then loaded into glass racks and rinsed in 1 X SSC for 30 sec, washed in 1 X SSC at 55°C for 60 min, rinsed in 1 X SSC (30 sec) and 0.1 X

SSC (30 sec) at room temperature, dehydrated in ethanol (70 and 95%; 30 sec each) and dried (Table 2.3). When dry, slides were apposed to X-ray film with standard [ $^{14}\text{C}$ ] microscaler in autoradiographic cassettes for between 3 and 5 weeks.

## 2.7 AUTORADIOGRAPHY

As the autoradiography protocols differ according to the ligand in use, each protocol will be described in the appropriate section in Chapter 4.

## 2.8 EMULSION DIPPING

Under darkroom conditions, nuclear emulsion (LM1; Amersham, UK) was diluted 1:1 with distilled water containing 1% glycerol in a specimen container. The emulsion solution was then heated in a water bath set at 42°C for approximately 60 min. The container was then carefully inverted to mix the contents into a homogeneous solution. To facilitate the dipping of microscope slides or coverslips, the emulsion solution was then transferred to a triangular perspex container and maintained at 42°C.

### 2.8.1 ISHH

Slide-mounted brain tissue sections were dipped individually in the emulsion solution, placed into a cardboard box and allowed to dry in a sealed, light-tight box. On the following day, the slides that had been dipped in photographic emulsion were transferred to microscope slide boxes containing Silica Gel desiccant. These boxes were then wrapped completely in foil and stored at 4°C for between 12 and 20 weeks, depending on the length of time that the sections were previously apposed to X-ray film. Slides were then developed as in section 2.9.2.

## 2.8.2 AUTORADIOGRAPHY

Full slide-length coverslips were loaded into glass racks and soaked overnight in caustic detergent (R.B.S.). The coverslips were then rinsed thoroughly in tap water and distilled water and subsequently dried in the oven at 40°C. Under darkroom conditions, the coverslips were individually dipped in photographic emulsion, pegged to a horizontal wire and left overnight to dry in a light-tight box. The next day, coverslips were glued (Supa-glue) to the frosted end of the slides so that the coverslip also covered the brain sections. Small metal clips also held the coverslips in place. Coverslipped slides were then placed into large boxes containing Silica gel desiccant, wrapped in foil and stored at 4°C for the appropriate length of time. Slides were then developed as in section 2.9.2.

## 2.9 PHOTOGRAPHIC DEVELOPMENT

### 2.9.1 X-RAY AND TRITIUM-SENSITIVE FILMS

X-ray films were processed and developed using an automatic X-ray Film Processor (100 Plus; All-Pro Imaging Corp, USA).

Tritium-sensitive films were developed manually under darkroom conditions. Films were submerged in Kodak D19 (4 min), transferred to a stop solution (1 min), fixed in a 1:4 solution of Hypam Rapid Paper and Film fixer (3 min), washed in distilled water (1 min), rinsed in distilled water containing Photo-Flo (0.01%; 1 min) and hung up to dry.

### 2.9.2 EMULSION-DIPPED SLIDES AND COVERSGLIPS

The same protocol was used for the development of emulsion-dipped slides and emulsion-dipped coverslips that had been apposed to slide-mounted sections. Emulsion-dipped slides or coverslips were developed in Kodak D19 (2 min), rinsed in distilled water (5-10 sec), fixed

(Hypam Rapid Paper and Film Fixer; 3 min), and washed in distilled water (5-10 sec). Brain sections were then prepared for light microscopy as described in section 2.10.

## 2.10 PREPARATION OF SECTIONS FOR LIGHT MICROSCOPY

After photographic development, brain sections were stained in 0.1% thionin, rinsed in distilled water, differentiated in serial alcohol dilutions (70%, 90% and 3 X 100%) and cleared in HistoClear®. The sections were either coverslipped (ISHH) or sealed (autoradiography) using DPX® mountant. Individual brain sections were examined under a light microscope (Olympus BH-2) using both light- and dark-field condensers.

Brain sections collected for use as a reference set for ISHH, autoradiography or immunohistochemistry were stained, differentiated, cleared and coverslipped as described above.

## 2.11 PHOTOGRAPHY AND IMAGING

An Olympus C-35AD camera attached to the light microscope (Olympus BH-2) was used to take both black and white (Ilford FP4+) and colour (Kodak Gold) photographs. Scanned and photographed images were produced with the assistance of Medical Illustration and Richard Crompton, Faculties of Medicine and Science, Monash University. All of the schematic brain maps used in figures throughout the thesis were adapted from (Paxinos & Watson, 1986).

## 2.12 IMAGE ANALYSIS

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Quantitative densitometric analysis of the autoradiographic images was performed on an MCID (Micro Computing Imaging Device) M4 image analysis system (Imaging Research Inc., Canada) connected to an IBM compatible computer. Images were visualised using a lightbox providing a constant level of illumination (Northern Light; Imaging Research Inc., Canada). Film images were captured with a Sony XC-77CE CD video camera attached to a Nikon Micro-Nikkor lens (55mm) and displayed on a 15" colour monitor.

The density of hybridisation signal or receptor binding was assessed from film images by comparing the optical density of the autoradiograms to a calibration curve obtained from the analysis of standard [ $^{14}\text{C}$ ] or [ $^3\text{H}$ ] microscales. For bilateral nuclei, left and right values were averaged for each section. Brain nuclei were defined by comparison with adjacent counterstained sections and with reference to a stereotaxic atlas of the rat brain (Paxinos & Watson, 1986).

## 2.13 STATISTICAL ANALYSIS

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The statistical program, GraphPad Prism®, was used for all analyses. A number of different statistical tests were used throughout the thesis, and these will be described in more detail in the appropriate chapters. In all cases, a level of  $P < 0.05$  was considered to be statistically significant.

## 2.14 MATERIALS

### 2.14.1 GENERAL

[ <sup>14</sup> C] microscapes	American Radiolabeled Chemicals Inc., USA
[ <sup>3</sup> H] sensitive film (Hyperfilm)	Amersham, UK
[ <sup>3</sup> H] microscapes	Amersham, UK
DPX mountant	BDH Laboratory Supplies, UK
Gelatine	BDH Laboratory Supplies, UK
Histoclear	National Diagnostics, USA
Hypam rapid paper and film fixer	Ilford, Australia
Kodak D19	Kodak, USA
Microscope slides	Objektträger, Germany
Naloxone	Sigma Chemical Co., Australia
Photographic emulsion (LM-1)	Amersham, UK
Sodium methohexitone (Brietal Sodium)	Lilly, Australia
Sodium pentobarbitone (Nembutal)	Boehringer Mannheim, Germany
Stop solution	Kodak, USA
X-ray film (X-OMAT)	Kodak, USA

## 2.14.2 ISHH

[ $\alpha$ - <sup>33</sup> P]dATP	NEN, DuPont, USA
Chloroform	Biolab Scientific, Australia
Cobalt chloride	Boehringer Mannheim, Germany
Dextran sulfate sodium	Pharmacia Biotech, Sweden
Diethyl pyrocarbonate	Sigma Chemical Co., Australia
Formamide, deionised	Fluka, Switzerland
Paraformaldehyde	Merck, Australia
Poly-L-lysine	Sigma Chemical Co., Australia
Prepro-ENK oligonucleotide probe *	Dr Gundlach (Melb. Uni) or Oligo service, Biochemistry Dept., Monash Uni
Prepro-GAL oligonucleotide probes *	Life Technologies, Australia
Prepro-NPY oligonucleotide probes *	Biotech International Ltd., WA
ProDYN oligonucleotide probes *	CyberSyn, USA
Sephadex G25 (medium)	Pharmacia Biotech, Sweden
Terminal deoxynucleotidyl transferase	Boehringer Mannheim, Germany
Terminal transferase reaction buffer	Boehringer Mannheim, Germany

\*: The sequence of each oligonucleotide probe(s) used to visualise prepro-ENK, proDYN, prepro-GAL or prepro-NPY mRNA is shown in section 3.2.3.

## 2.14.3 AUTORADIOGRAPHY

[ <sup>125</sup> I]-GAL; rat	NEN, DuPont, USA
[ <sup>3</sup> H]-naltrindole	NEN, DuPont, USA
[ <sup>3</sup> H]-U69-593	NEN, DuPont, USA
Bovine Serum Albumin	Commonwealth Serum Laboratories, Australia
FK 33-824	Bachem, Germany
Galanin; rat	American Peptide Co., Inc.
Na[ <sup>125</sup> I]	Amersham, UK
Supa-glue	Selleys

## 2.14.4 IMMUNOHISTOCHEMISTRY

3',3'-diaminobenzidine tablets	Sigma Chemical Co., Australia
Glucose oxidase	Sigma Chemical Co., Australia
Normal rabbit serum	Life Technologies, Australia
Rabbit anti-sheep biotinylated secondary antibody	Silenus, Australia
Sheep polyclonal anti-Fos antiserum	Genosys, England
Streptavidin-conjugated horseradish peroxidase	Silenus, Australia

# IN SITU HYBRIDISATION HISTOCHEMISTRY



## Histochemistry

**His''to-kem'is-tre**

Noun - that branch of histology which deals with the identification of chemical components in cells or tissues.

# **CHAPTER 3**

## **IN SITU HYBRIDISATION HISTOCHEMISTRY**

### **3.1 INTRODUCTION**

The use of ISHH to investigate the central distribution of neuropeptides was first reported in 1985 with the detection of the mRNA encoding vasopressin (Nojiri *et al.*, 1985). Since this time, mRNA encoding neuropeptide precursors such as prepro-ENK (Harlan *et al.*, 1987), proDYN (Morris *et al.*, 1986), prepro-GAL (Ryan & Gundlach, 1996), prepro-NPY (Morris, 1989) and POMC (Larsen & Mau, 1994) have been detected in the CNS and peripheral tissues. The distribution of mRNA encoding a variety of receptors has also been reported (Mansour *et al.*, 1994a). Thus, ISHH has been developed as a valuable tool that can be used to provide detailed information regarding the regulation of the synthesis of the mRNA encoding numerous peptides and receptors. Moreover, knowledge of the mechanisms underlying regulation of mRNA synthesis can assist with studies of the role of these specific neuropeptides within the CNS and peripheral tissues.

Techniques such as northern blot analysis can also determine the mRNA content of various tissues. Northern blot analysis involves the extraction of mRNA from large tissue areas, addition of the appropriate cDNA probe and subsequent mRNA separation on a gel, such that the results are expressed as mRNA content within a gross region such as the hypothalamus. While this provides useful region to region comparisons, it cannot delineate the mRNA content of specific subnuclei within gross anatomical regions. ISHH assists in the quantification of the mRNA content of discrete regions on tissue sections, thus providing a visual representation of the regional distribution of mRNA for a specific gene on the section. The use of serial sections can also provide information on rostro-caudal differences in the levels of mRNA within a region. Furthermore, experimentally-induced alterations in mRNA expression can be examined within discrete central nuclei using ISHH, whereas the use of northern blot analysis in these experiments only permits gross comparisons, and subtle changes in mRNA expression confined to subnuclei are often hidden and undetectable. Although not utilised in this thesis, ISHH using

multiple probes labeled with different fluorophores in conjunction with confocal microscopy enables visualisation of the colocalisation of the mRNA for different genes within the same neurons. Therefore, the use of tissue sections is a clear advantage associated with the use of ISHH, as a substantial amount of information regarding the mRNA distribution and degree of expression within specific subnuclei can be obtained from one experiment.

In the following chapter, a standard ISHH procedure (Wisden & Morris, 1993; McLean *et al.*, 1996) using radiolabeled oligonucleotide probes that have been directed at the mRNAs encoding prepro-ENK, proDYN, prepro-GAL and prepro-NPY will be employed to visualise the distribution of these mRNAs throughout the normotensive and hypertensive rat CNS. Neurochemical alterations have been previously reported in the basal state of hypertensive rats compared to normotensive controls (see section 1.4.2), including differences in central opioid, GAL and NPY systems. McLean and co-workers demonstrated the presence of basal differences in the central expression of prepro-NPY in SHR compared to WKY, while the Yin laboratory have compared the expression of the three major opioid peptide precursors in the CNS of WKY and SHR (McLean *et al.*, 1996; Yin *et al.*, 1996b; Yin *et al.*, 1997). Moreover, there are no reports that have compared the basal levels of prepro-GAL mRNA content through multiple levels of the CNS of SHR and WKY. Thus, a comparison of the basal levels of expression of the various neuropeptide precursor mRNAs between strains will provide further information regarding central neural changes in the hypertensive state. Furthermore, as exposure to stress results in a complex neurochemical response, this chapter will provide an insight into the plastic and dynamic regulation of peptide synthesis in the brain following acute and chronic restraint. Hypertensive rats have been reported to exhibit an altered physiological and behavioural stress response compared to normotensive rats (see section 1.4.4), and the underlying neurochemical bases for these differences are not clear. To assist in the explanation of these differences in the stress response, the effect of restraint on the synthesis of the mRNA encoding prepro-ENK, proDYN, prepro-GAL and prepro-NPY in the CNS will be compared between normotensive and hypertensive rat strains in this chapter.

## 3.2 METHODS

### 3.2.1 RESTRAINT PARADIGM

For the details regarding animals and ethics, refer to sections 2.1 and 2.2. For a general description of the restraint paradigm, refer to section 2.3. Rats from both strains (WKY and SHR) were subjected daily to 60 min restraint stress for 1, 3, 5 or 10 consecutive days (n=3 rats per strain for each restraint period). Rats in the control group (0 sessions of stress; n=3 per strain) were transferred to the laboratory, remained in their home cage for 1.5 hours (i.e. the same time in the laboratory as the stressed rats) and immediately killed as described below. The use of 3 rats in each group represents a significant number of subjects, given the use of 2 different rat strains and 5 separate restraint groups. While larger subject groups may have reduced variability and strengthened the data, the size of the ISHH experiments would have been impractical. As described in a later section (section 3.2.3.5), the ISHH experiments were designed and conducted in a way to reduce the effects of experimental variation and provide an equal platform for comparisons of the effects of restraint and/or strain on gene expression.

The protocol for each day of restraint stress was identical, with rats being exposed to restraint at the same time each day between 10:00h and 12:00h. After the restraint period, each rat was released from the tube and either transferred to its home cage to await continuation of the restraint paradigm on the following day or immediately killed by decapitation. Following decapitation, brains were dissected and removed, frozen over liquid nitrogen and stored at -80°C until further use.

### 3.2.2 PREPARATION OF SLIDE-MOUNTED BRAIN SECTIONS

For the collection of brain sections from both WKY and SHR rats, refer to section 2.5. Sections were thaw-mounted onto poly-L-lysine-coated microscope slides that were prepared according to the protocol described in section 2.5.1.

### 3.2.3 ISHH

The same protocol was used for each ISHH experiment, with the only difference being the oligonucleotide probe that was used. The protocol for pre-treatment of the sections prior to hybridisation, labeling of the oligonucleotide probes(s) with [ $\alpha$ - $^{33}$ P]-dATP, hybridisation procedure and washing of the brain sections is described in detail in section 2.6. The characteristics of each oligonucleotide probe are described below.

#### 3.2.3.1 *Prepro-ENK mRNA*

The 45-mer antisense synthetic cDNA oligonucleotide probe to prepro-ENK was obtained from either Dr Gundlach (University of Melbourne) or from the Oligo service, Biochemistry Department, Monash University. The oligonucleotide probe was complementary to nucleotides 259-303 of the rat prepro-ENK cDNA (sequence 5' CCT CCA TTG GCC TCT TCT TCT GGC TCC ACG GGG TAA AGC TCA TCC 3') (Yoshikawa *et al.*, 1989).

#### 3.2.3.2 *ProDYN mRNA*

A combination of 45-mer cDNA oligonucleotide probes was used to assess proDYN mRNA levels, and these probes were obtained from CyberSyn, PA, USA. The first probe was complementary to bases 860-904 of rat proDYN cDNA (sequence 5' CCC ACT TAA GCT TGG GGC GAA TGC GCC GCA GGA AGC CCC CAT AGC 3') (Civello *et al.*, 1985), and the second oligonucleotide was complimentary to bases 421-465 of rat proDYN cDNA (sequence 5' AAA AGA CCA AAA GCC CCG GCA TGT CTC CCA CTC CTC CGA GGG CGG 3') (Civello *et al.*, 1985).

#### 3.2.3.3 *Prepro-GAL mRNA*

Two 45-mer antisense synthetic cDNA oligonucleotide probes to prepro-GAL were obtained from Life Technologies, Australia. The first oligonucleotide was complementary to nucleotides

259-303 of the GAL peptide coding region of rat prepro-GAL cDNA (sequence 5' TGA TCT GTG GTT GTC AAT GGC ATG TGG GCC CAG AAG GTA GCC AGC 3') (Ryan & Gundlach, 1996). The second oligonucleotide was complimentary to bases 472-516 of the GAL peptide C-terminal portion of rat prepro-GAL cDNA (sequence 5' GGA CTG CTC TAG GTC TTC TGA GGA GGT GGC CAA GGG GAT GCC AGG 3') (Ryan & Gundlach, 1996).

#### 3.2.3.4 *Prepro-NPY mRNA*

The two oligonucleotide probes used to visualise expression of prepro-NPY mRNA in the rat CNS consisted of 48 nucleotides and were purchased from Biotech International Ltd., Western Australia. The first probe was complementary to nucleotides 3146-3194 of the rat NPY gene (sequence 5' ATG AGA TGT GGG GGG AAA CTA GGA AAA GTC AGG AGA GCA AGT TTC ATT 3') (Larhammar *et al.*, 1987; Pieribone *et al.*, 1992), while the second oligonucleotide probe was complementary to nucleotides 1629-1676 of the rat NPY gene (sequence 5' GTC CTC TGC TGG CGC GTC CTC GCC CGG ATT GTC CGG CTT GGA GGG GTA 3') (Mikkelsen *et al.*, 1994).

#### 3.2.3.5 *Specificity controls*

The same control conditions were used in each ISHH experiment. Control hybridisations were carried out in the presence of a 100-fold molar excess of either the unlabeled oligonucleotide combination (for proDYN, prepro-GAL and prepro-NPY) or the single oligonucleotide (for prepro-ENK). For each ISHH experiment, the presence of an excess of unlabeled oligonucleotide selectively abolished the hybridisation signal, suggesting that the signal was specific. In addition, all oligonucleotide probes yielded reproducible and consistent profiles of gene expression on adjacent tissue sections from different rat strains under identical experimental conditions. Another clear indicator of specific hybridisation for each oligonucleotide probe combination was the markedly different topographic profile throughout the CNS that was specific for each gene.

The specific activity of the labeled oligonucleotide probes/probe combinations varied from one experiment to another due to the inherent variations associated with enzymatic labeling.

Therefore, the ISHH experiments required careful preparation and planning to minimise the potential impact of this variation. Each ISHH experiment involved the simultaneous processing of multiple sections from one brain region only (forebrain, pons or medulla) from 30 rats (15 WKY and 15 SHR) for each probe on the same day, which constituted approximately 180 sections per experiment. Specifically, this protocol meant that 12 separate experiments were required for ISHH processing in this chapter (i.e. 3 regions x 4 neuropeptide probes/combinations). This protocol also ensured that quantitation of mRNA expression from the different rats enabled valid comparisons of the effects of restraint stress on the density of the signals obtained, since brain slices from each individual experiment were simultaneously processed from hybridisation through to photographic development of autoradiograms. Furthermore, this procedure is well established for quantitative analysis of mRNA expression (McLean *et al.*, 1996; Cowen *et al.*, 1998).

### 3.2.4 EMULSION PREPARATION AND DIPPING

After the three to five week period during which the brain sections were apposed to X-ray film, films were removed from the cassettes and processed automatically as described in section 2.9.1. A selection of slide-mounted brain sections from all ISHH experiments were then dipped in photographic emulsion according to the protocol described in sections 2.8 and 2.8.1 and ultimately developed as in section 2.9.2 after a period of 12-20 weeks.

### 3.2.5 IMAGE ANALYSIS AND STATISTICS

An MCID M4 image analysis system was utilised to analyse all autoradiograms generated by the ISHH experiments (section 2.12). Results were expressed as mean  $\pm$  standard error of the mean (S.E.M.), and the statistical program, GraphPad Prism®, was used for all statistical analyses. For each region that was analysed, three separate statistical tests were employed:

**BETWEEN STRAIN COMPARISON – BASAL EXPRESSION DIFFERENCES:** The unpaired student's *t*-test was used to determine significant differences in basal (day 0) neuropeptide precursor mRNA expression between strains, and if the data failed the test of equal variance, Welch's correction was used in addition to the unpaired student's *t*-test.

**WITHIN STRAIN COMPARISON – EFFECT OF RESTRAINT:** To determine statistically significant differences between neuropeptide precursor mRNA expression in control and stressed groups for each strain, a one way analysis of variance (ANOVA) followed by a post-hoc Dunnett's test was employed.

**BETWEEN STRAIN COMPARISON – EFFECT OF RESTRAINT:** A two way ANOVA was utilised to compare the temporal effect of restraint stress on the expression of neuropeptide precursor mRNA between WKY and SHR rats. Normalised data (relative to baseline levels of expression) were processed using a two way ANOVA to counteract any possible effect of different basal levels of expression, and hence different baselines, on the statistical comparison of the temporal response to restraint between strains. In situations where a significant interaction occurred between the number of restraint sessions and strain, subsequent post-hoc analysis was performed to identify the source of the variation.

In all cases, a level of  $P < 0.05$  was considered to be statistically significant.

### 3.2.6 MATERIALS AND ABBREVIATIONS

For all materials used in this chapter, refer to sections 2.14.1 and 2.14.2. For all abbreviations, refer to page xvii.

## 3.3 RESULTS

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### 3.3.1 BEHAVIOURAL CHANGES INDUCED BY RESTRAINT

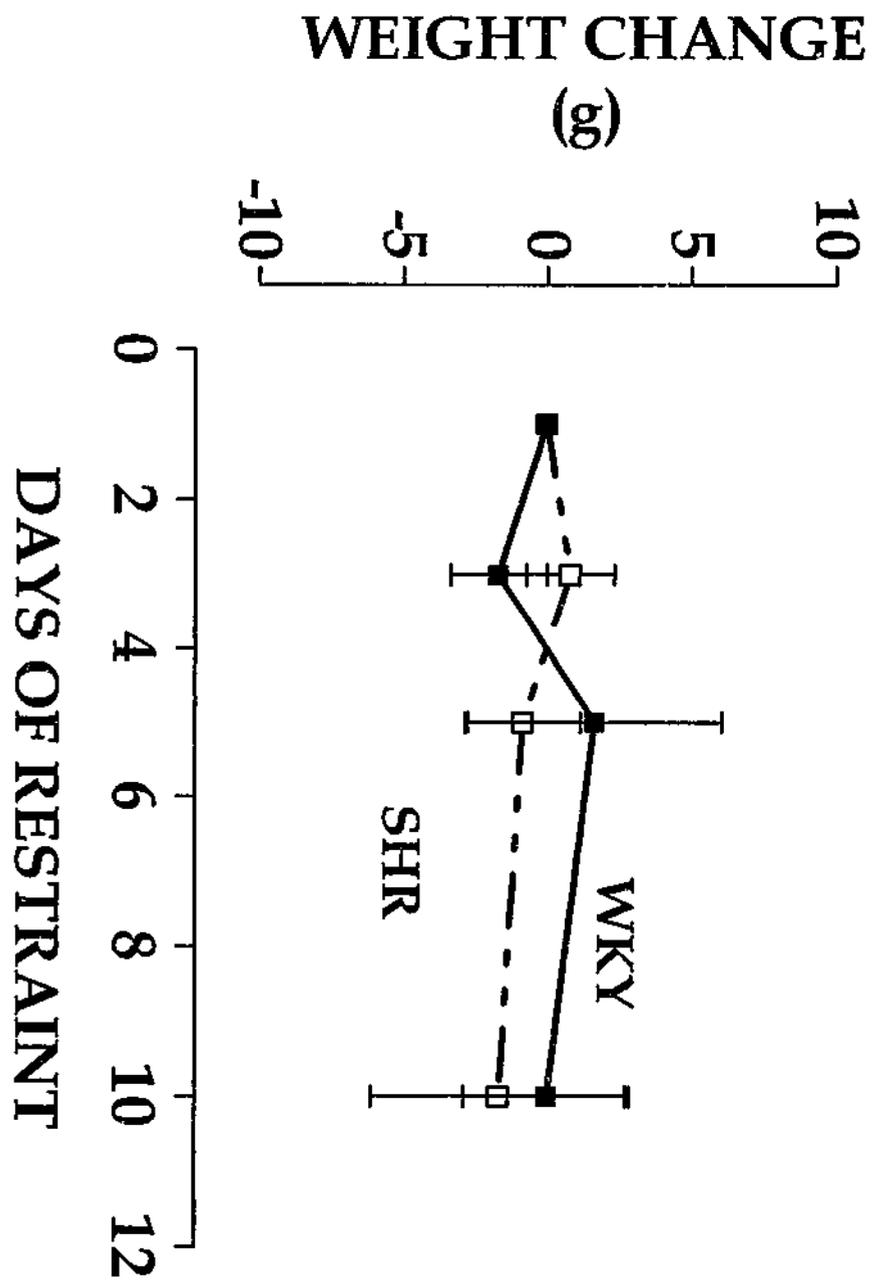
General behavioural changes in both strains that were qualitatively observed during the stress paradigm included increased urination, defaecation, respiration and attention during restraint, and increased activity once the rats were released from the restraint tube. As the number of restraint stress sessions increased, the urine and faecal output during the restraint period

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**FIGURE 3.1**

Changes in weight of WKY (filled boxes) and SHR (empty boxes) following exposure to a 60 min restraint stress for 0 (control), 1, 3, 5 and 10 consecutive days (n=3 rats per group per strain). Results are expressed as mean weight change (g)  $\pm$  S.E.M.

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decreased, suggesting the presence of habituation. The weights of rats from both strains did not vary significantly over the course of the stress paradigm (Figure 3.1).

### 3.3.2 PREPRO-ENK mRNA EXPRESSION

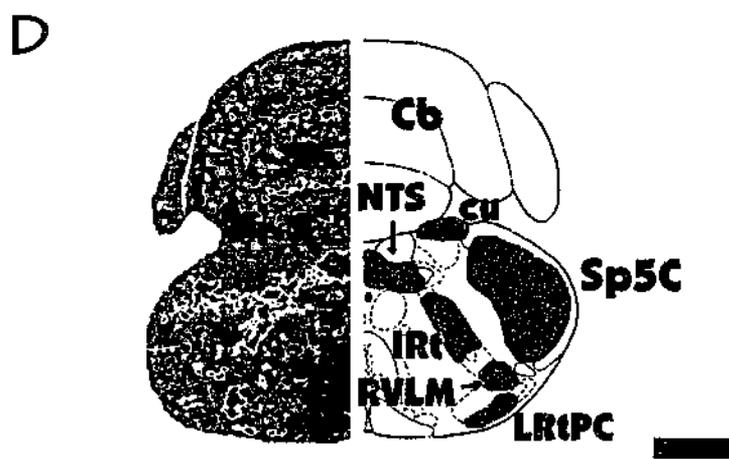
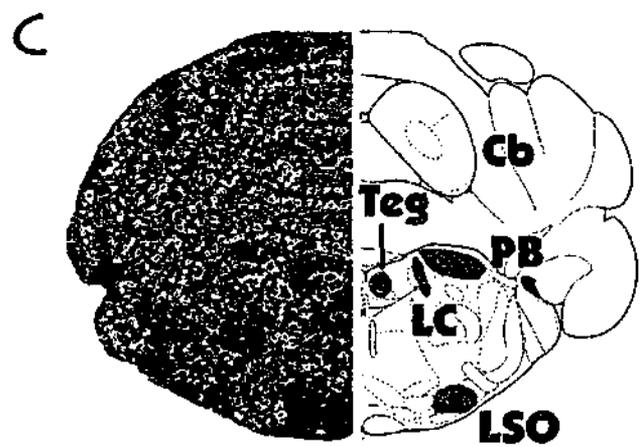
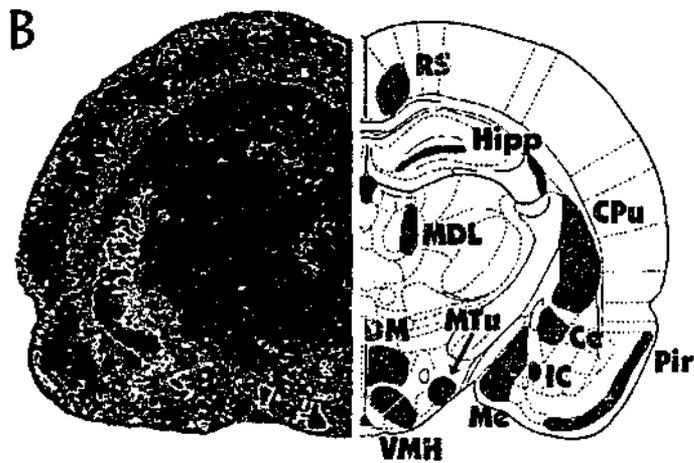
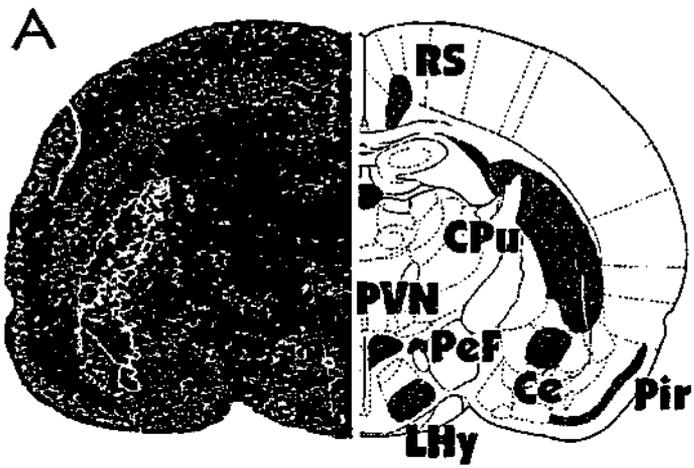
#### 3.3.2.1 Strain comparison

Prepro-ENK mRNA was found to be widely expressed throughout the CNS of both WKY and SHR, with transcript detected in nuclei such as the PVN and other hypothalamic subregions, amygdaloid nuclei, cortical regions (Cing, Pir), CPu, LC, cerebellum, NTS and RVLM. Regions such as the hippocampus, thalamus and the medullary reticular fields also contained prepro-ENK mRNA, but the level of the transcript was not quantified in these regions (Table 3.1). Representative pseudocolour autoradiograms with associated brain maps are shown in Figure 3.2. To obtain a more detailed cellular resolution of the distribution of prepro-ENK mRNA, slide-mounted sections were dipped in photographic emulsion. Following development, clusters of silver grains were detected over individual neurons in nuclei such as the NTS, RVLM and CPu (Figure 3.3).

Prepro-ENK mRNA was detected in similar regions throughout the CNS of both WKY and SHR, with a comparable distribution in the forebrain, pons and medulla (Figure 3.4). Following quantification of prepro-ENK mRNA hybridisation, the basal level of expression of the mRNA encoding prepro-ENK was compared between WKY and SHR (Figure 3.5). Prepro-ENK mRNA expression was significantly higher in the Tz (+53%), cerebellum (+58%) and RVLM (+28%) of SHR when compared to WKY ( $P < 0.05$ ; student's unpaired  $t$ -test). There were two additional regions where prepro-ENK mRNA levels were elevated in SHR compared to WKY, but the increases were not significant. The PeF, which is located lateral to the PVN, contained elevated levels of prepro-ENK mRNA ( $+54 \pm 20\%$ ;  $P = 0.087$ ; student's unpaired  $t$ -test with Welch's correction) in SHR compared to WKY, while a marked, but non-significant increase of  $+114 \pm 52\%$  in prepro-ENK mRNA expression was detected in the SHR LC compared to WKY ( $P = 0.055$ ; student's unpaired  $t$ -test).

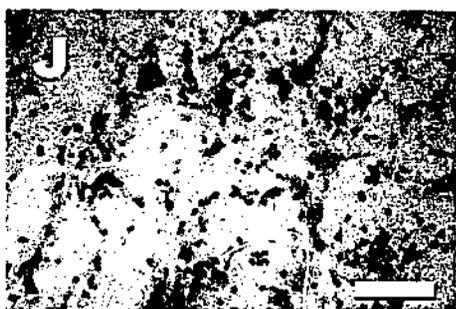
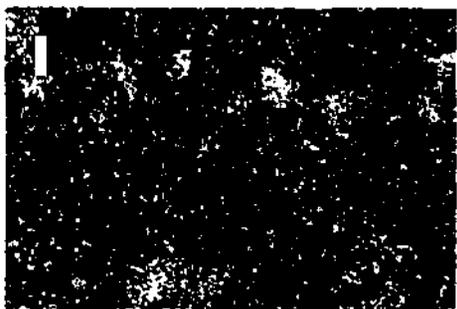
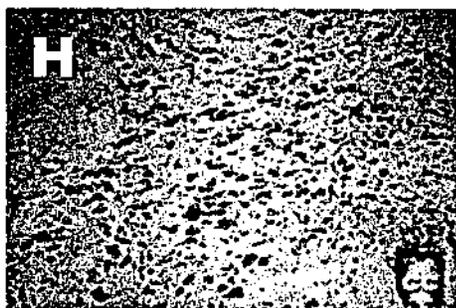
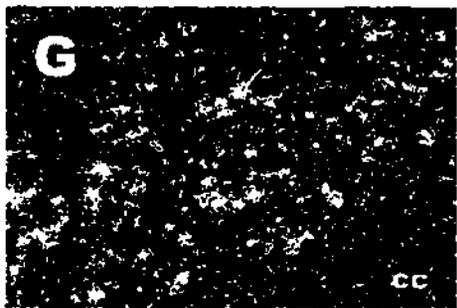
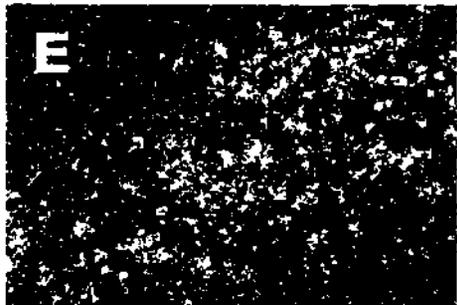
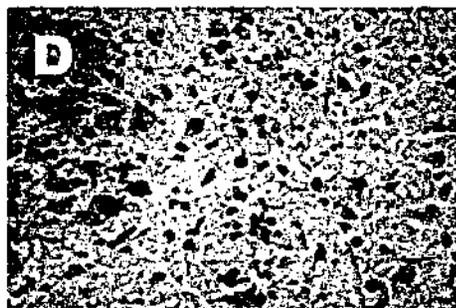
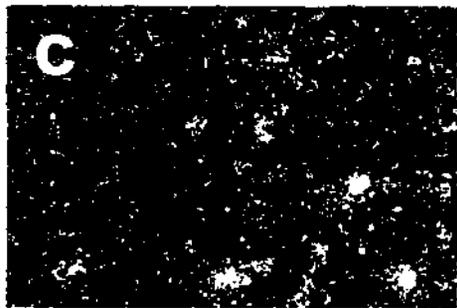
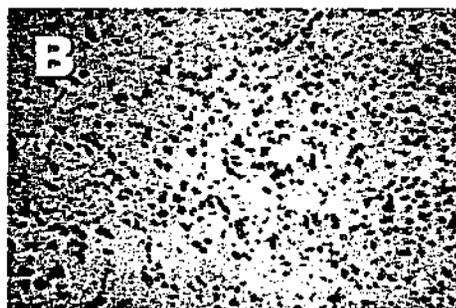
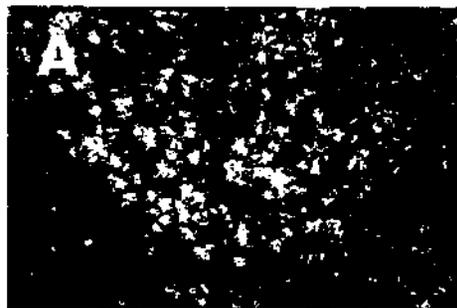
**FIGURE 3.2**

Representative pseudocolour autoradiograms and corresponding brain maps demonstrating the distribution of prepro-ENK mRNA hybridisation at four levels of the rat CNS (A – forebrain: bregma ~ -1.8mm; B – forebrain: bregma ~ -3.1mm; C – pons: – bregma ~ -9.7mm; D – medulla oblongata: bregma ~ -14.1mm). For abbreviations, refer to page xvii. Scale bar represents 1.88mm (A and B), 1.45mm (C) and 1.38mm (D).



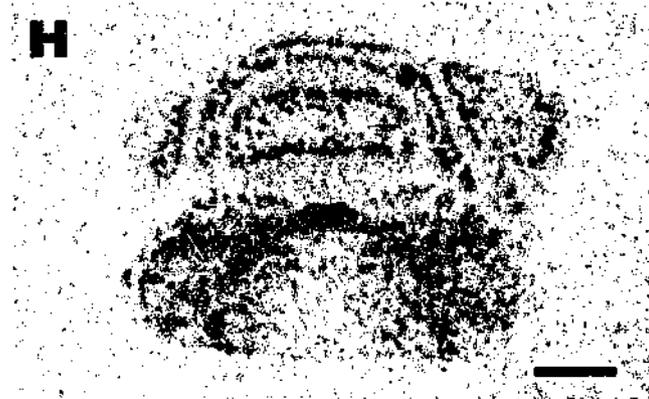
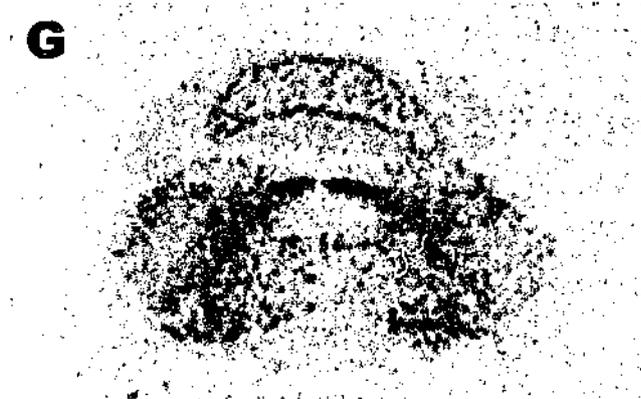
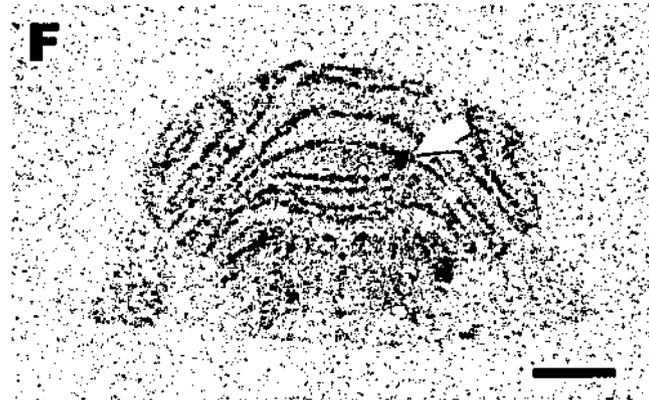
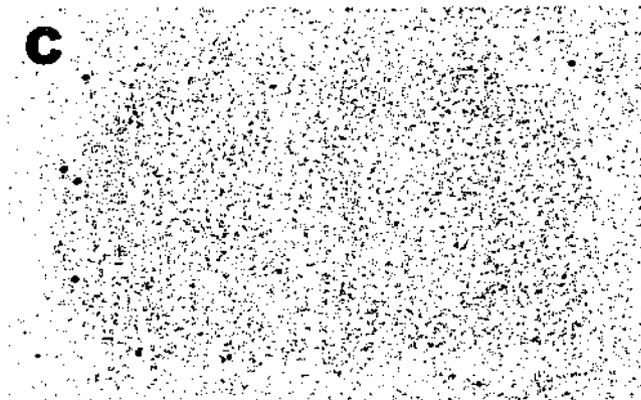
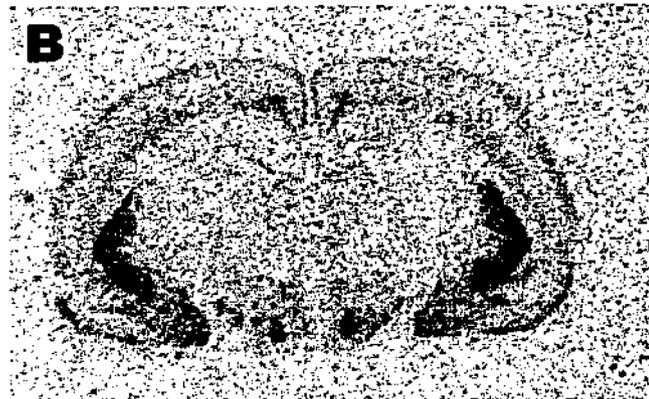
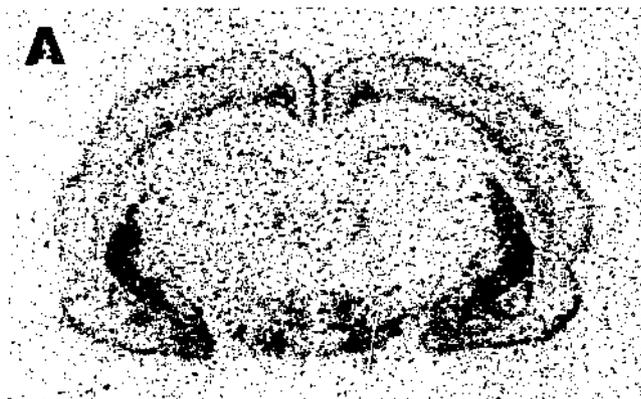
**FIGURE 3.3**

Dark-field (A, C, E, G, I) and corresponding light-field (B, D, F, H, J) photomicrographs of emulsion-dipped slides demonstrating the presence of perikarya expressing prepro-ENK mRNA in various brain nuclei (A, B – CPu; C, D – PeF; E, F – PB; G, H – commissural NTS; I, J – RVLM). Silver grains represent the presence of prepro-ENK mRNA. Scale bar represents 200 $\mu$ m (A, B, E, F, G, H) or 100 $\mu$ m (C, D, I, J). For abbreviations, see page xvii.



**FIGURE 3.4**

Autoradiographic images of prepro-ENK mRNA hybridisation in the forebrain (A-D), pons (E, F) and medulla oblongata (G, H) of normotensive (WKY; A, C, E, G) and hypertensive (SHR; B, D, F, H) rat brain. Panels C and D represent the non-specific hybridisation of prepro-ENK in the forebrain of WKY and SHR respectively, which is indicative of the extremely low level of non-specific hybridisation observed throughout the CNS of both strains. White arrow points to artifact resulting from the ISHH procedure. Scale bar represents 2.42mm (A-D), 2.28mm (E, F) or 1.33mm (G, H).



**FIGURE 3.5**

Comparison of basal (no stress) prepro-ENK mRNA hybridisation density in the CNS of normotensive (WKY) and hypertensive (SHR) rat strains (n=3 per strain). Density of prepro-ENK mRNA hybridisation is expressed as the % change in SHR compared to WKY. For abbreviations, see page xvii.

\*:  $P < 0.05$ ; unpaired student's *t*-test.

# % CHANGE IN SHR COMPARED TO WKY

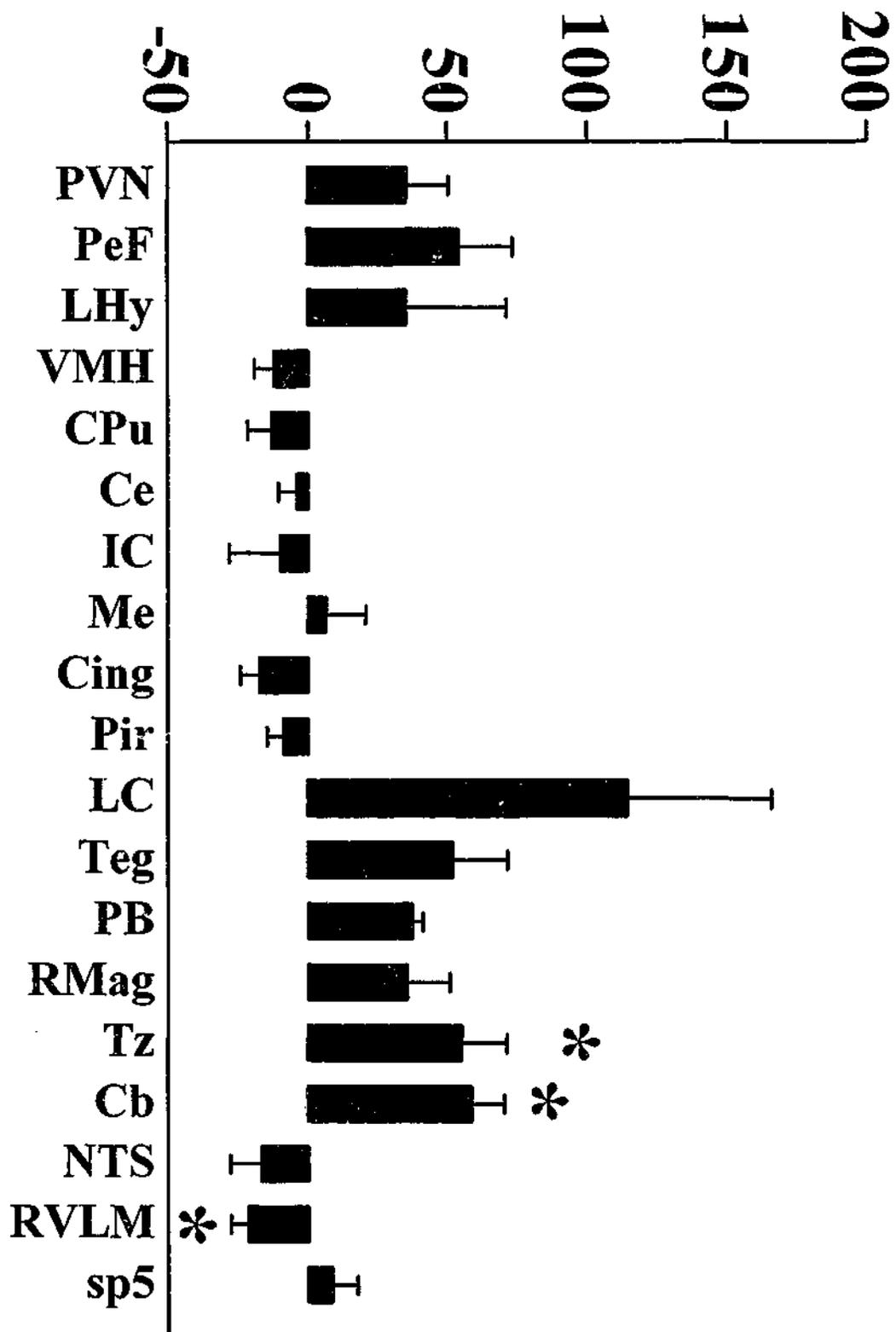


TABLE 3.1

REGION	RELATIVE DENSITY OF prepro-ENK mRNA	
	WKY	SHR
<b>FOREBRAIN</b>		
PVN	+	++
PeF	++	+++
DM	+	+
LHy	++	+++
VMH	+++	+++
MTu	++	++
CPu	+++	+++
Hipp	+	+
Ce	+++	+++
IC	+++	+++
Me	+	+
MDL	+	+
RS	+	+
Pir	+	+
<b>PONS</b>		
LC	+	++
Teg	++	+++
PB	+	++
SubC	+	+
RMag	++	++
Tz	++	+++
LSO	+	+
Cb	+	+
<b>MEDULLA</b>		
NTS	+++	+++
sp5	+++	+++
RVLM	+++	+++
cu	+	+
IOC	++	++
Rt	++	++

Relative distribution of prepro-ENK mRNA in the CNS of WKY and SHR rats. The density of prepro-ENK mRNA hybridisation was assessed as follows: +++ - dense ( $> 5$  DPM/mm<sup>2</sup>); ++ - moderate (2 DPM/mm<sup>2</sup> to 5 DPM/mm<sup>2</sup>) and + - light ( $< 2$  DPM/mm<sup>2</sup>). For abbreviations, see page xvii.

### 3.3.2.2 Restraint stress and prepro-ENK mRNA expression

Acute and chronic restraint stress induced significant changes in prepro-ENK mRNA expression in the CNS of both strains of rats used in the present study. In the hypothalamus, significant changes were detected in a number of subregions. In WKY, acute restraint induced a significant increase in prepro-ENK mRNA levels in the PeF (+126%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test) which returned towards baseline levels following further exposure to restraint. In contrast, no significant changes were observed in the PeF of SHR throughout the restraint paradigm. Comparison of the temporal response to restraint between WKY and SHR in the PeF confirmed that the response to this stressor was significantly different between strains ( $F(4,20) = 2.75$ ;  $P < 0.05$ ; two way ANOVA) (Figure 3.6B). Interestingly, the level of prepro-ENK mRNA within the PVN was not altered by acute or chronic restraint in either strain (Figure 3.6A). The LH<sub>y</sub> was sensitive to perturbation by restraint in both strains. In WKY, chronic (10 sessions) restraint resulted in a significant decrease (-43%;  $P < 0.05$ ), while acute (-60%;  $P < 0.01$ ) and chronic periods of restraint (-60%; 5 periods and -56%; 10 periods; both  $P < 0.05$ ) induced significantly reduced expression of prepro-ENK mRNA in the SHR LH<sub>y</sub> (one way ANOVA with post-hoc Dunnett's test). The hypothalamic VMH contained the highest level of prepro-ENK mRNA expression in the forebrain, and within this region chronic restraint produced a significantly elevated level of prepro-ENK mRNA in SHR (+70% (3 sessions); +35% (10 sessions); both  $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test) (Figure 3.6C). However, the restraint paradigm used in the current study had no effect on prepro-ENK mRNA expression in the VMH of WKY, suggesting that the VMH within WKY and SHR may respond differently to restraint stress. Further support was provided by the results of a two way ANOVA comparison, which demonstrated the presence of a significant interaction between strain and periods of exposure to restraint ( $F(4,20) = 2.81$ ;  $P < 0.05$ ; two way ANOVA).

The CPu of WKY exhibited a response to the restraint paradigm that may be indicative of adaptation, with an initial significant increase in prepro-ENK mRNA after 1 period of restraint (+40%;  $P < 0.05$ ), followed by a decrease to basal levels of expression on subsequent days of restraint exposure (Figure 3.6D). In SHR, a different response pattern was observed, with a significant increase in prepro-ENK mRNA expression after 3 (+78%;  $P < 0.01$ ) and 10 periods of restraint (+47%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). Furthermore, statistical comparison using a two way ANOVA showed that the temporal response of neurons containing prepro-ENK mRNA within the CPu was significantly different between WKY and

**FIGURE 3.6**

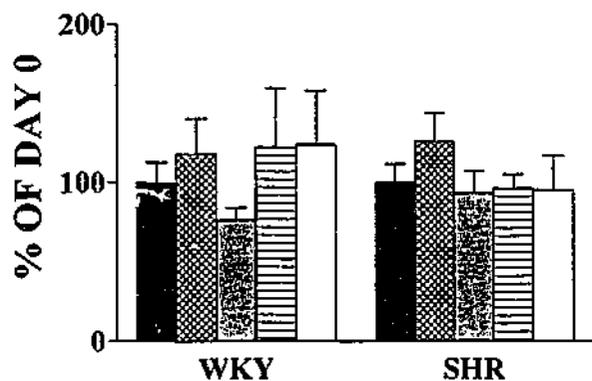
The effect of acute and chronic restraint stress on the density of prepro-ENK mRNA hybridisation in selected forebrain regions of normotensive (WKY) and hypertensive (SHR) rat CNS. Rats were exposed to a 60 min period of restraint stress for 0 (control; filled black columns), 1 (hatched columns), 3 (filled grey columns), 5 (horizontal lined columns) or 10 (open columns) consecutive days (n=3 rats per group per strain). Brain regions represented are: A – PVN; B – PeF; C – VMH; D – CPu; E – Ce and F – Pir. Results are expressed as a percentage of day 0 (control) levels  $\pm$  S.E.M. For abbreviations, refer to page xvii.

\*:  $P < 0.05$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

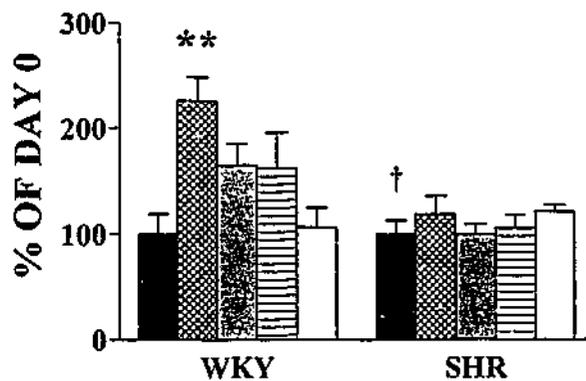
\*\* :  $P < 0.01$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

†:  $P < 0.05$  when comparing the temporal response to restraint in SHR with WKY; two way ANOVA.

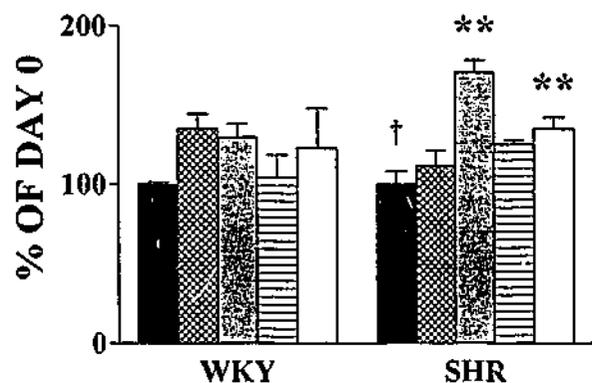
### A - PVN



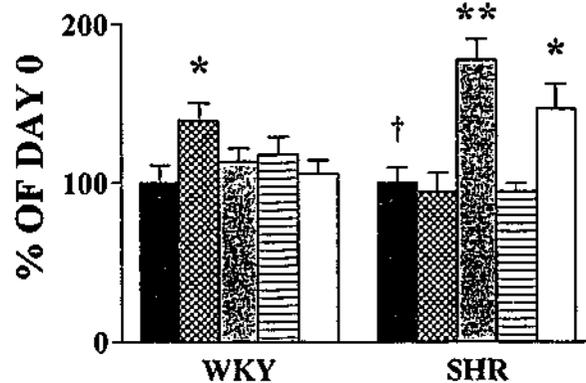
### B - PeF



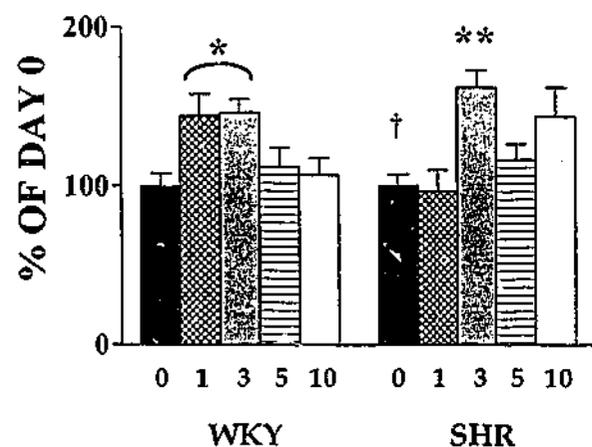
### C - VMH



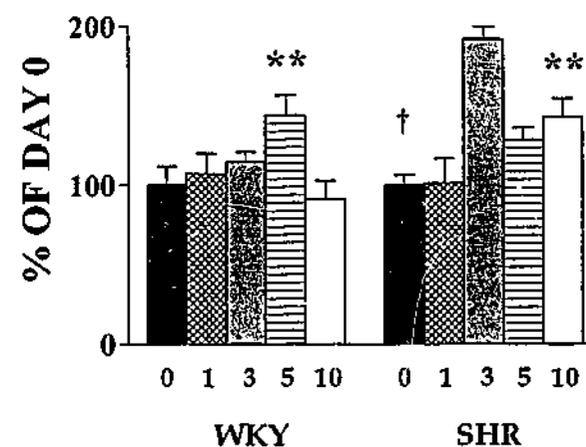
### D - CPu



### E - Ce



### F - Pir



SHR ( $F(4,20) = 8.36$ ;  $P < 0.001$ ; two way ANOVA). The levels of prepro-ENK were also significantly increased in the Ce of both strains, with a  $\sim +45\%$  elevation after 1 and 3 sessions of restraint in WKY ( $P < 0.05$ ), and a 62% increase in SHR following 3 periods of restraint ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test). Interestingly, the temporal response to restraint stress was significantly different between strains at the level of the Ce ( $F(4,20) = 1.2$ ;  $P < 0.05$ ; two way ANOVA), and visual comparison of the graphs (Figure 3.6E) clearly shows that the prepro-ENK mRNA-containing neurons within the WKY Ce respond preferentially to acute restraint, while in the SHR the Ce neurons containing prepro-ENK were activated by 3 consecutive periods of restraint.

An interesting response pattern was observed in the LC of WKY and SHR (Figure 3.7A). Exposure to the 10 day restraint paradigm produced a bell-shaped response, with a significant change in prepro-ENK mRNA levels observed in both strains on day 3 of the restraint paradigm. In WKY, prepro-ENK mRNA expression was significantly increased (+148%;  $P < 0.05$ ), while in contrast in SHR, prepro-ENK mRNA expression decreased significantly by -59% ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). The difference in the temporal nature of the response of the LC to restraint between SHR and WKY was confirmed using a two way ANOVA ( $F(4,20) = 4.23$ ;  $P < 0.01$ ). Moreover, while the prepro-ENK mRNA-containing neurons in the LC of SHR and WKY have a quite different response to restraint stress, the fact that prepro-ENK mRNA expression returns towards baseline levels after 5 and 10 days of the restraint paradigm regardless of the direction of the transcriptional change suggests that adaptation to the stressor may be occurring in both strains. Another pontine region, the Tz, exhibited significant decreases in expression of the prepro-ENK transcript in response to the restraint paradigm, with decreases on day 1 (-42%;  $P < 0.01$ ) and day 5 (-37%;  $P < 0.01$ ) of the restraint paradigm in WKY and decreases on days 1 (-52%;  $P < 0.01$ ), 3 (-27%;  $P < 0.05$ ), 5 (-50%;  $P < 0.01$ ) and 10 (-39%;  $P < 0.01$ ) of the restraint paradigm in SHR (one way ANOVA with post-hoc Dunnett's test).

The level of expression of prepro-ENK mRNA was reduced by restraint in the cerebellum of both strains (Figure 3.7B). In WKY, 1, 3 and 5 periods of restraint produced a significant decrease of approximately -22% ( $P < 0.01$ ). In addition, in the cerebellum of SHR, the significant reductions in prepro-ENK mRNA were of a two-fold greater magnitude than WKY and these were sustained for the entire 10 day restraint paradigm (between -44% and -56%; sessions 1, 3, 5 and 10;  $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test). A statistical comparison of the stress response within the cerebellum of WKY and SHR confirmed that strain

**FIGURE 3.7**

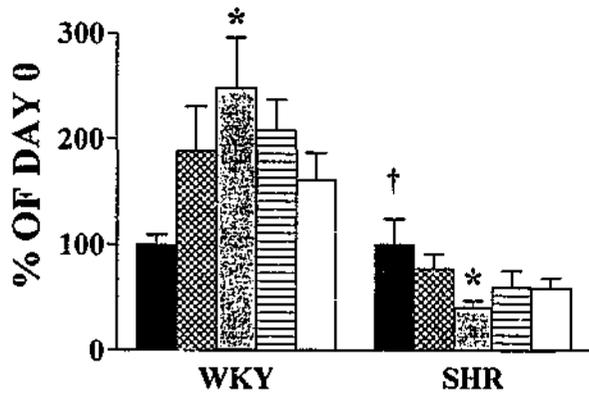
The effect of acute and chronic restraint stress on the density of prepro-ENK mRNA hybridisation in selected pontine and medullary regions of normotensive (WKY) and hypertensive (SHR) rat CNS. Rats were exposed to a 60 min period of restraint stress for 0 (control; filled black columns), 1 (hatched columns), 3 (filled grey columns), 5 (horizontal lined columns) or 10 (open columns) consecutive days (n=3 rats per group per strain). Brain regions represented are: A – LC; B – Cb; C – NTS and D – RVLM. Results are expressed as a percentage of day 0 (control) levels  $\pm$  S.E.M. For abbreviations, refer to page xvii.

\*:  $P < 0.05$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

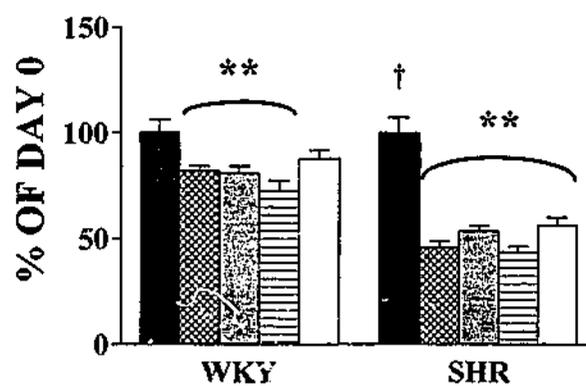
\*\* :  $P < 0.01$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

†:  $P < 0.05$  when comparing the temporal response to restraint in SHR with WKY; two way ANOVA.

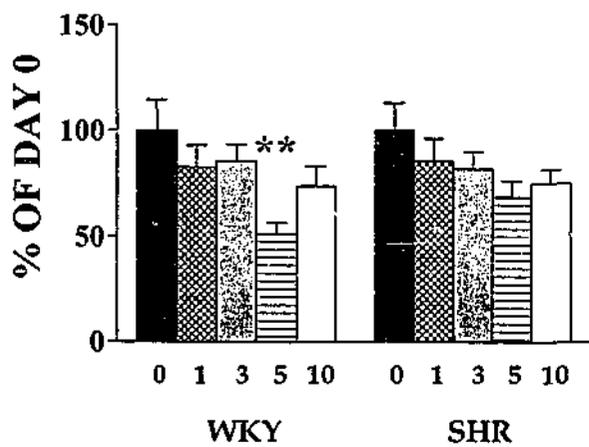
### A - LC



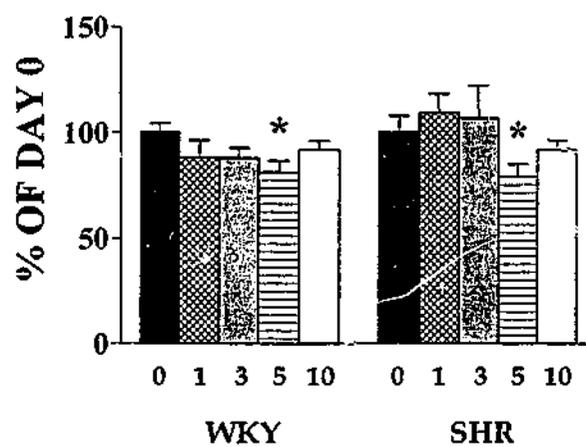
### B - Cb



### C - NTS



### D - RVLM



did influence the prepro-ENK mRNA response to restraint within this region ( $F(4,20) = 5.47$ ;  $P < 0.001$ ; two way ANOVA).

In the NTS and RVLM, the level of prepro-ENK mRNA was relatively high compared to other central regions. While prepro-ENK mRNA levels were not altered by restraint in the NTS of SHR, 5 sessions of restraint produced a significant decrease of almost -50% in the WKY NTS ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test) (Figure 3.7C). When comparing both WKY and SHR, a similar response to restraint was observed in the RVLM, as 5 periods of restraint reduced prepro-ENK mRNA levels by ~ -20% ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test) (Figure 3.7D).

### 3.3.3 PRODYN mRNA EXPRESSION

#### 3.3.3.1 Strain comparison

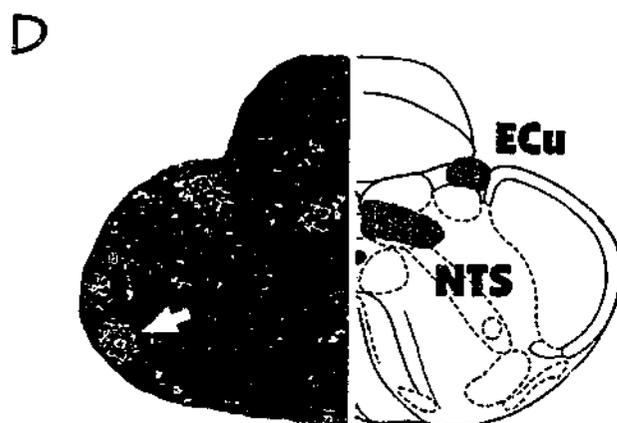
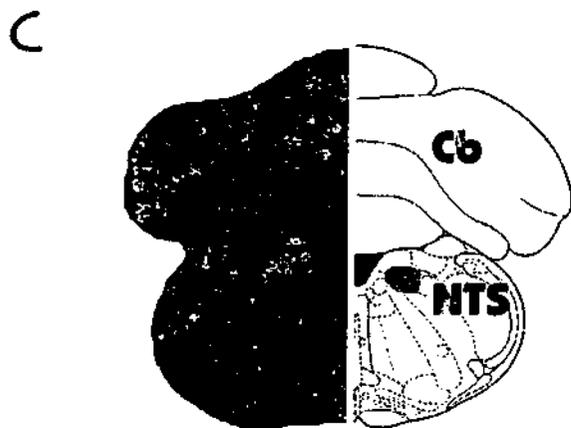
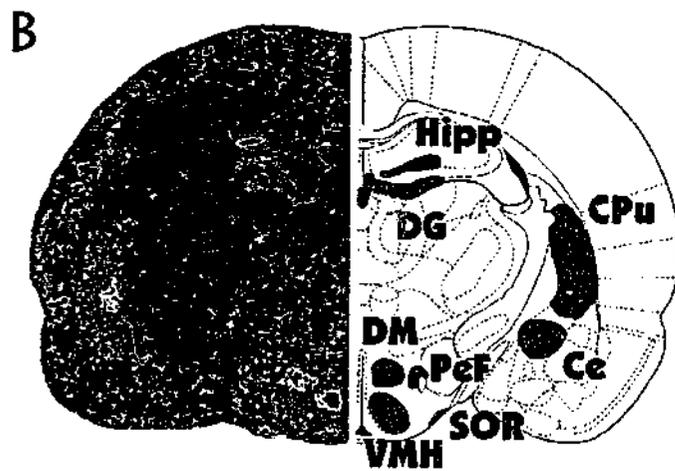
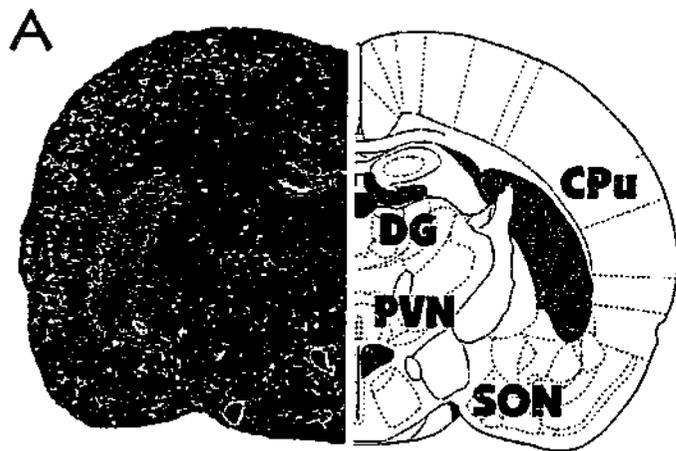
ProDYN mRNA was also detected throughout the CNS of WKY and SHR, with expression of proDYN confined to brain nuclei such as the DG, PVN, SON, CPu, Ce and NTS (Figure 3.8). Moreover, the distribution of proDYN mRNA was similar between strains (Figure 3.9). As shown in Table 3.2, the distribution of proDYN is a great deal more restricted than the widespread populations of neurons containing prepro-ENK. In addition to the regions where the level of proDYN mRNA expression was quantified, the Par and LH<sub>y</sub> also contained detectable levels of proDYN mRNA (see Table 3.2). Comparison of the basal levels of expression of the precursor for the dynorphins revealed a number of regions where the expression of the proDYN transcript was significantly reduced in SHR compared to WKY, including the SON (-59%), DG (-73%), CPu (-28%) and NTS (-42%) ( $P < 0.05$ ; student's unpaired *t*-test) (Figure 3.10).

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**FIGURE 3.8**

Representative pseudocolour autoradiograms and corresponding brain maps demonstrating the distribution of proDYN mRNA hybridisation at four levels of the rat CNS (A – forebrain: bregma ~ -1.8mm; B – forebrain: bregma ~ -3.1mm; C – medulla oblongata: bregma ~ -12.8mm; D – medulla oblongata: bregma ~ -14.3mm). For abbreviations, refer to page xvii. White arrow represents a random artifact that was a result of the ISHH procedure. Scale bar represents 1.88mm (A and B), 1.54mm (C) and 1.08mm (D).

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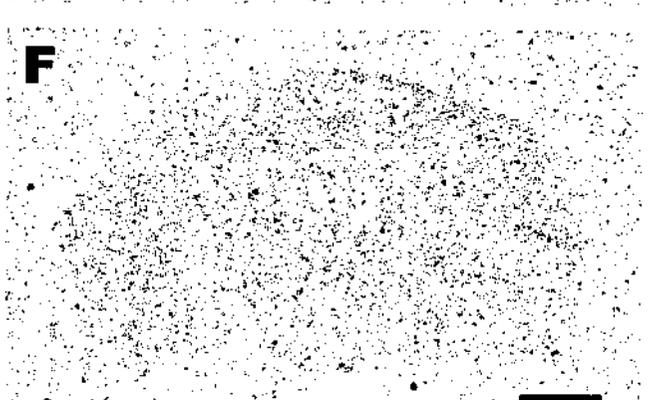
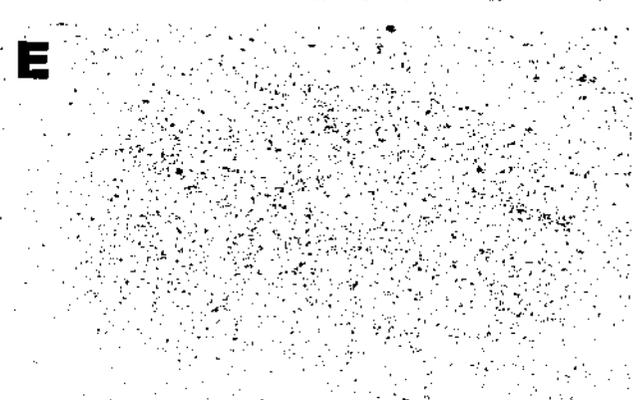
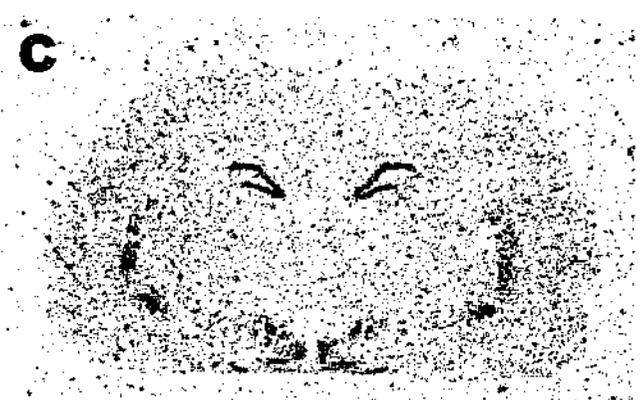
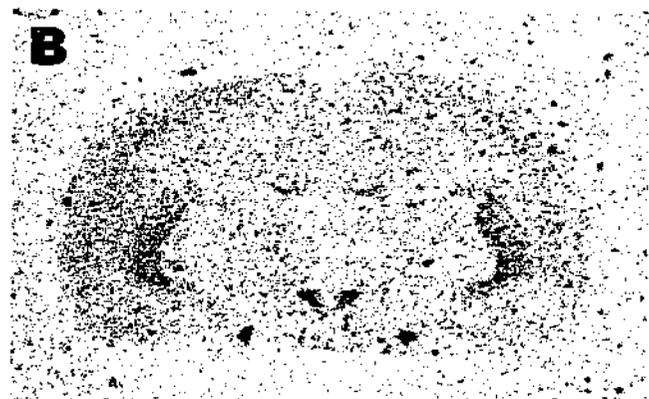
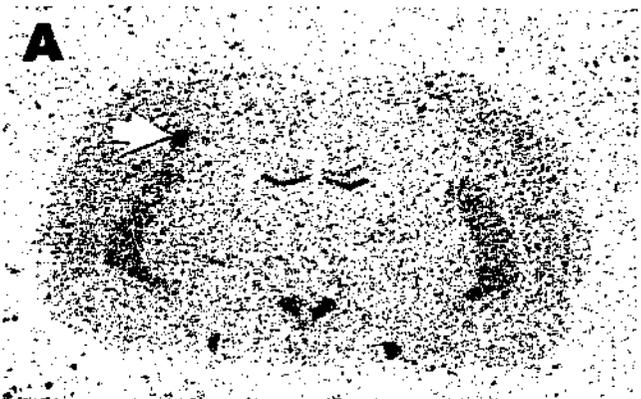


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**FIGURE 3.9**

Autoradiographic images of proDYN mRNA hybridisation in the forebrain (A-F), and medulla oblongata (G, H) of normotensive (WKY; A, C, E, G) and hypertensive (SHR; B, D, F, H) rat brain. Panels E and F represent the non-specific hybridisation of proDYN in the forebrain of WKY and SHR respectively, which is indicative of the extremely low level of non-specific hybridisation observed throughout the CNS of both strains. White arrows point to artifacts resulting from the ISHH procedure. Scale bar represents 2.42mm (A-F) or 1.33mm (G, H).

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**FIGURE 3.10**

Comparison of basal (no stress) proDYN mRNA hybridisation density in the CNS of normotensive (WKY) and hypertensive (SHR) rat strains (n=3 per strain). Density of proDYN mRNA hybridisation is expressed as the % change in SHR compared to WKY. For abbreviations, see page xvii.

\*:  $P < 0.05$ ; unpaired student's *t*-test.

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# % CHANGE IN SHR COMPARED TO WKY

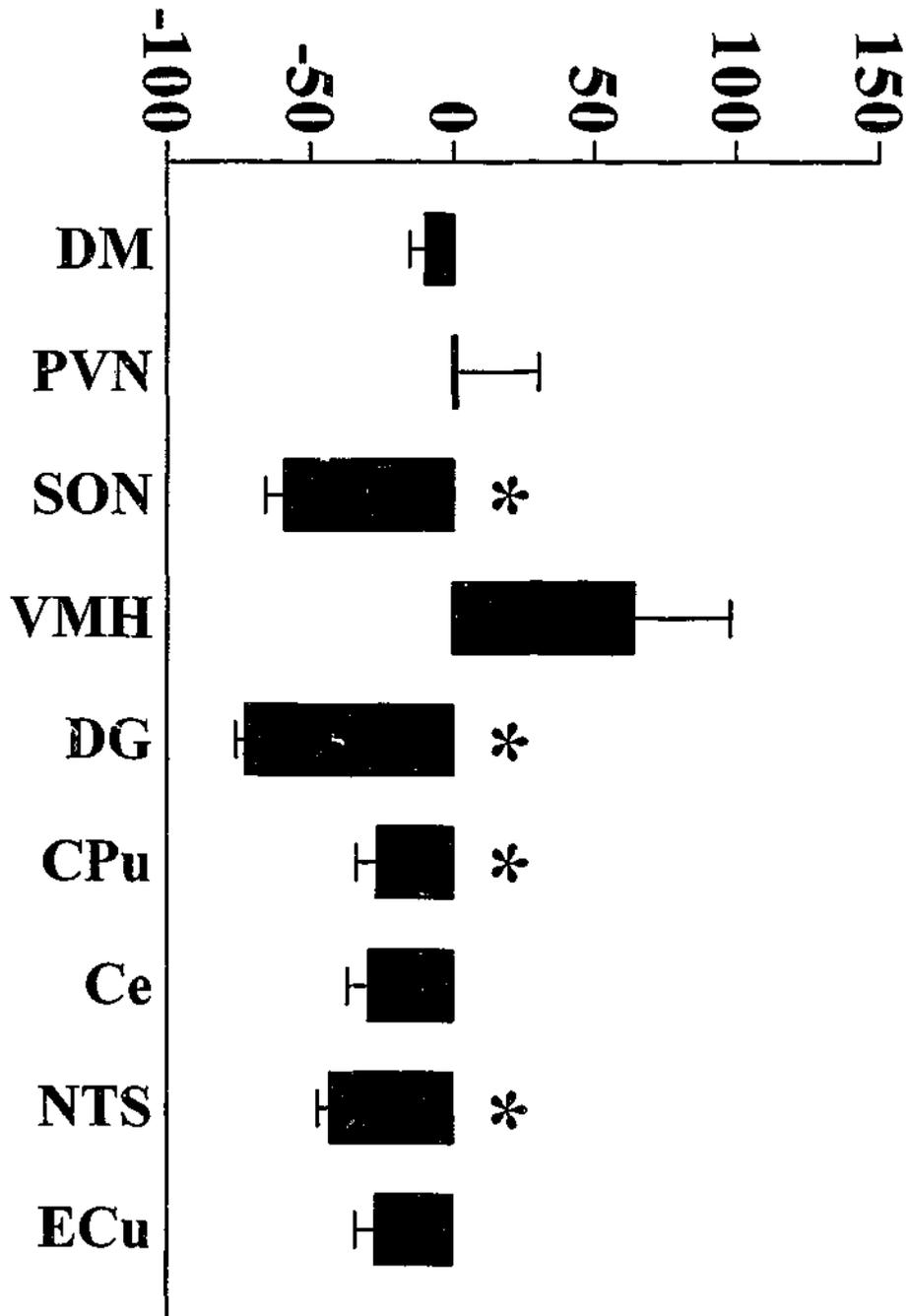


TABLE 3.2

REGION	RELATIVE DENSITY OF proDYN mRNA	
	WKY	SHR
<b>FOREBRAIN</b>		
DM	++	++
LHy	++	++
PVN	+++	+++
SON	+++	++
VMH	++	++
DG	++	+
CPu	+	+
Ce	++	++
Par	+	+
<b>MEDULLA</b>		
NTS	++	+
ECu	+	+

Relative distribution of proDYN mRNA in the CNS of WKY and SHR rats. The density of proDYN mRNA hybridisation was assessed as follows: +++ - dense ( $> 0.8$  DPM/mm<sup>2</sup>); ++ - moderate (0.3 DPM/mm<sup>2</sup> to 0.8 DPM/mm<sup>2</sup>) and + - light ( $< 0.3$  DPM/mm<sup>2</sup>). For abbreviations, see page xvii.

### 3.3.3.2 Restraint stress and proDYN mRNA expression

ProDYN mRNA was detected and quantified in several hypothalamic subregions, including the PVN, SON and VMH, and all of these regions displayed a different response to the restraint stress paradigm (Figure 3.11). In the SON of WKY, there were no changes in proDYN mRNA expression following either acute or chronic restraint stress. In contrast, neurons within the SON of SHR contained increased levels of proDYN mRNA in response to all periods of restraint stress, with the largest increase of +263% observed after 3 sessions of restraint ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test) (Figure 3.11A). The results of a two way ANOVA, designed to compare the temporal response to restraint in SHR and WKY, clearly shows that the

**FIGURE 3.11**

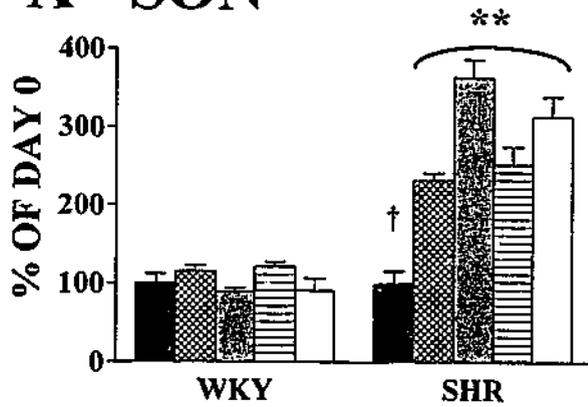
The effect of acute and chronic restraint stress on the density of proDYN mRNA hybridisation in selected regions of normotensive (WKY) and hypertensive (SHR) rat CNS. Rats were exposed to a 60 min period of restraint stress for 0 (control; filled black columns), 1 (hatched columns), 3 (filled grey columns), 5 (horizontal lined columns) or 10 (open columns) consecutive days (n=3 rats per group per strain). Brain regions represented are: A – SON; B – VMH; C – DG; D – CPu; E – Ce and F – NTS. Results are expressed as a percentage of day 0 (control) levels  $\pm$  S.E.M. For abbreviations, refer to page xvii.

\*:  $P < 0.05$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

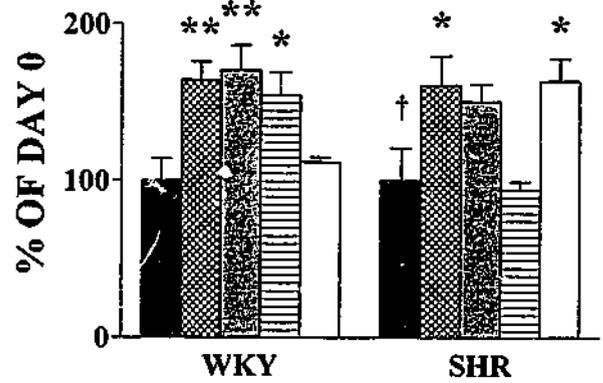
\*\* :  $P < 0.01$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

†:  $P < 0.05$  when comparing the temporal response to restraint in SHR with WKY; two way ANOVA.

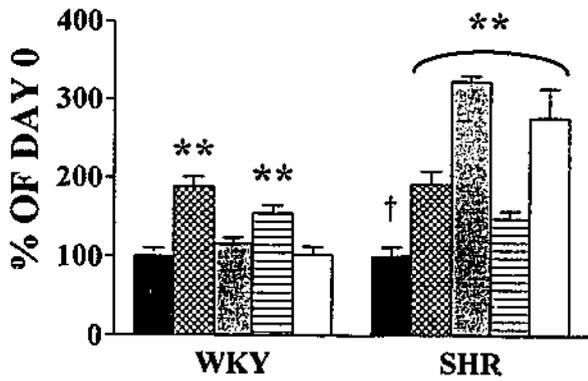
### A - SON



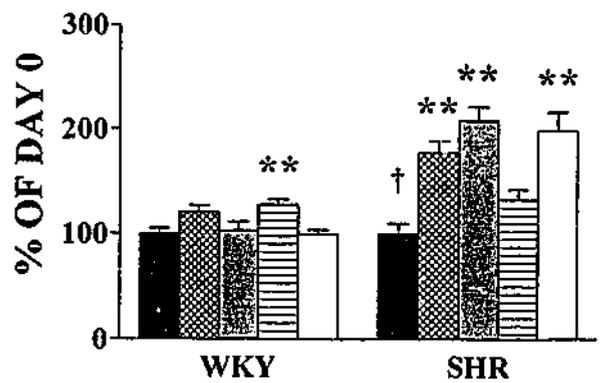
### B - VMH



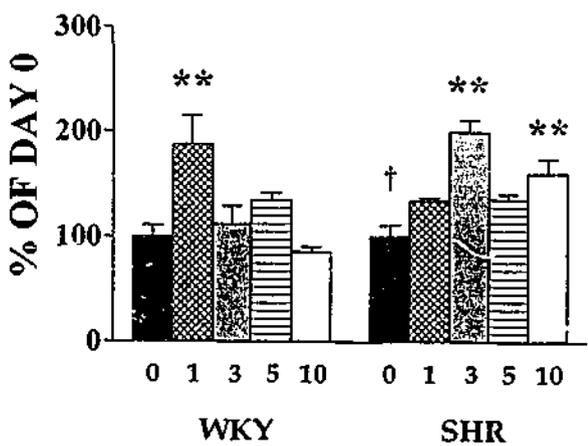
### C - DG



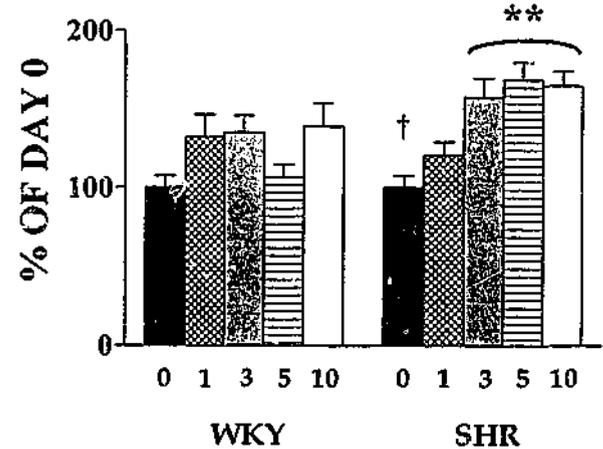
### D - CPu



### E - Ce



### F - NTS



genetic strain significantly influenced the response to the stressor in this study ( $F(4,20) = 18.72$ ;  $P < 0.001$ ; two way ANOVA).

The VMH was another hypothalamic nucleus where proDYN mRNA expression induced by restraint was significantly different between WKY and SHR ( $F(4,20) = 3.46$ ;  $P < 0.01$ ; two way ANOVA). Specifically, proDYN mRNA expression in the VMH of WKY increased after 1 day (+64%;  $P < 0.01$ ), 3 days (+70%;  $P < 0.01$ ) and 5 days (+55%;  $P < 0.05$ ) of the restraint paradigm and returned towards basal levels after 10 periods of restraint. In the SHR VMH, 1 day (+61%;  $P < 0.05$ ) and 10 days (+63%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test) of the restraint paradigm also produced a significantly elevated expression of proDYN mRNA, while 3 periods of restraint induced a non-significant increase of +51% (Figure 3.11B).

The DG contained high levels of expression of proDYN mRNA, and restraint stress elicited increases in proDYN mRNA expression in both strains. In the DG of WKY, the largest increase (+88%;  $P < 0.01$ ) in proDYN mRNA was detected after an acute (1 period) exposure to restraint. Both acute and chronic restraint induced significant elevations in proDYN mRNA expression in the DG of SHR, with increases of +91% (session 1;  $P < 0.01$ ), +223% (session 3;  $P < 0.01$ ), +48% (session 5;  $P < 0.01$ ) and +177% (session 10;  $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test) detected in this region (Figure 3.11C). A significant interaction between strain and number of days of restraint stress was subsequently found in the DG ( $F(4,20) = 18.7$ ;  $P < 0.001$ ; two way ANOVA).

The restraint stress paradigm also elicited reproducible increases in proDYN mRNA expression in the CPu of SHR, with significantly elevated levels of expression of approximately +180% to +210% after 1, 3 and 10 days of the restraint paradigm ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test) (Figure 3.11D). However, the neurons containing proDYN mRNA within the CPu of WKY were not as responsive to restraint stress, with 5 periods of restraint inducing a modest increase of +28% ( $P < 0.05$ ). Such a clear difference is reflected in the results of a two way ANOVA comparison, demonstrating that WKY and SHR exhibit a differential temporal response to restraint at the level of the CPu ( $F(4,20) = 13.15$ ;  $P < 0.001$ ; two way ANOVA).

The Ce, which also contained proDYN mRNA, displayed a significantly different temporal response to restraint stress in WKY and SHR ( $F(4,20) = 8.82$ ;  $P < 0.001$ ; two way ANOVA). The Ce in WKY appeared to respond primarily to acute restraint, with a significant increase of +87% after 1 session of restraint ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test)

(Figure 3.11E). Further exposure to restraint did not result in any significant increases in proDYN mRNA expression. In contrast, the proDYN mRNA-containing neurons in the Ce of SHR responded at a much slower rate to restraint. After 3 sessions of restraint, a significant increase of +99% ( $P < 0.01$ ) was observed, followed by another significant increase after 10 restraint exposures (+60%;  $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test).

Similarly, in the NTS of SHR, chronic restraint produced significant elevations in proDYN transcript levels, with 3, 5 and 10 days of the restraint paradigm eliciting increases of +58%, +69% and +65% respectively ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test) (Figure 3.11F). In contrast, the restraint paradigm did not alter proDYN mRNA expression in the NTS of the normotensive rat strain. Furthermore, a significant interaction between strain and the proDYN response to the 10 day restraint paradigm existed in this region ( $F(4,20) = 3.4$ ;  $P < 0.05$ ; two way ANOVA).

### 3.3.4 PREPRO-GAL mRNA EXPRESSION

#### 3.3.4.1 Strain comparison

Prepro-GAL mRNA was detected in several nuclei in the WKY and SHR CNS, including hypothalamic nuclei such as the PVN and SON, the cerebellum, LC, RVLM and NTS at the level of the AP (Table 3.3). Included in Table 3.3 are regions where prepro-GAL mRNA expression was detected but not quantified, such as some of the hypothalamic subregions (Pe, DM, LH). Examples of autoradiograms at the level of the medulla, pons and forebrain are shown in Figure 3.12. A more detailed cellular resolution of the distribution of prepro-GAL mRNA in each region was obtained using slide-mounted sections that had been dipped in photographic emulsion. As shown in Figure 3.13, clusters of silver grains were detected over individual neurons in regions such as the Ce, PVN and LC.

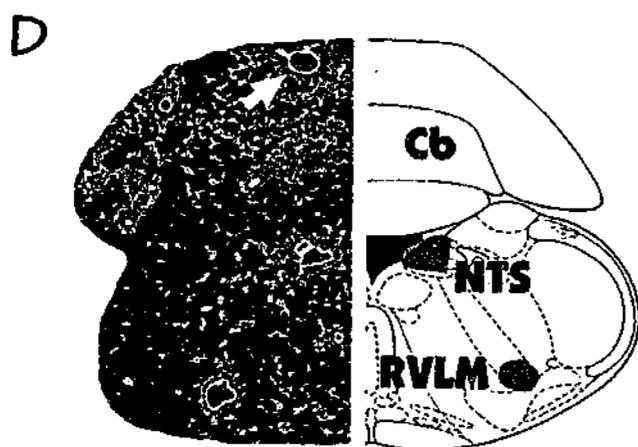
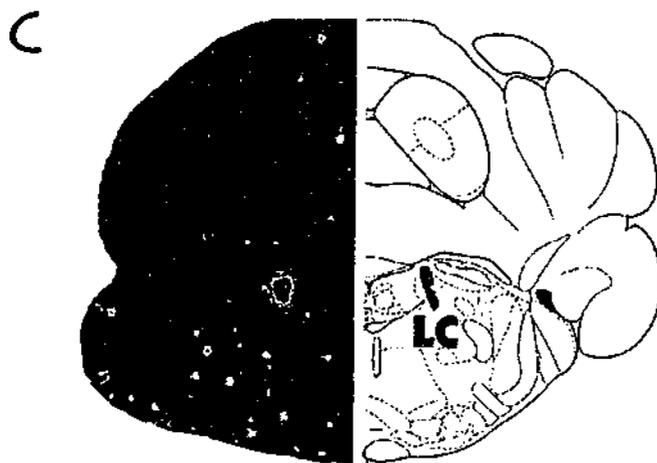
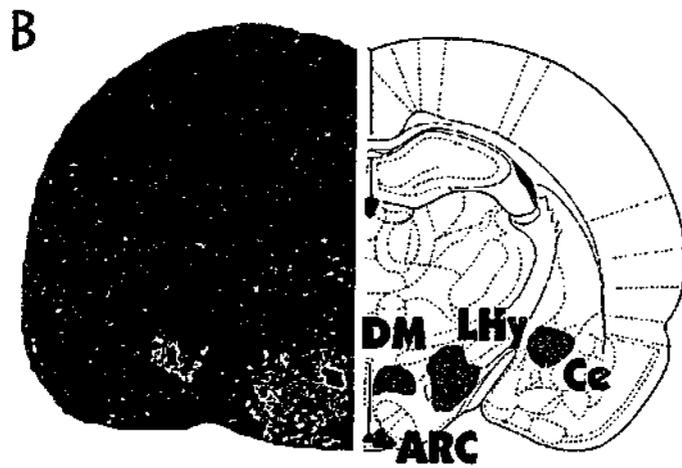
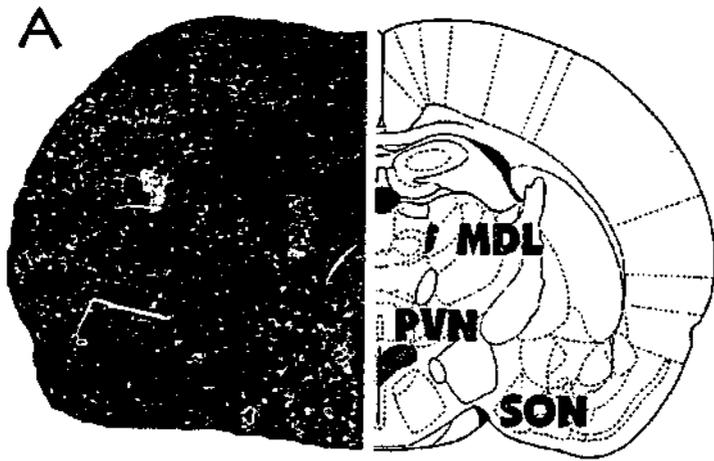
Figure 3.14 shows that prepro-GAL mRNA was found in identical regions in the CNS of both WKY and SHR. Following quantification, prepro-GAL mRNA expression was compared between WKY and SHR strains in control (unstressed) groups (Figure 3.15). Significant differences between strains were observed in the SON (-47%;  $P < 0.01$ , unpaired student's *t*-test) and in the RVLM of SHR (+207%;  $P < 0.05$ ; unpaired student's *t*-test with Welch's correction), when compared to WKY.

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**FIGURE 3.12**

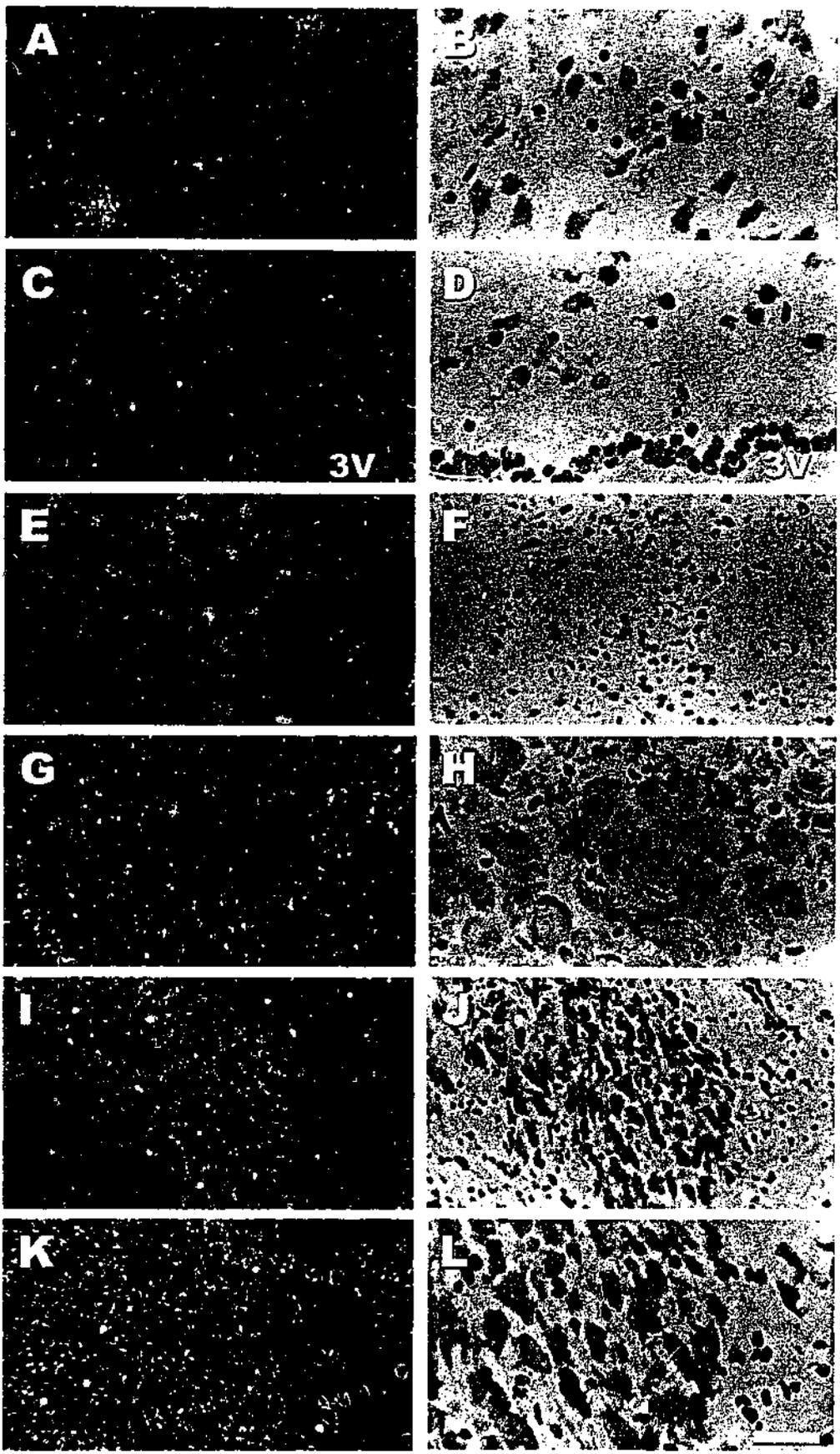
Representative pseudocolour autoradiograms and corresponding brain maps demonstrating the distribution of prepro-GAL mRNA hybridisation at four levels of the rat CNS (A – forebrain: bregma ~ -1.8mm; B – forebrain: bregma ~ -2.8mm; C – pons: bregma ~ -9.7mm; D – medulla oblongata: bregma ~ -13.3mm). White arrow points to a random artifact resulting from the ISHH procedure. For abbreviations, refer to page xvii. Scale bar represents 1.88mm (A and B), 1.45mm (C) and 1.08mm (D).

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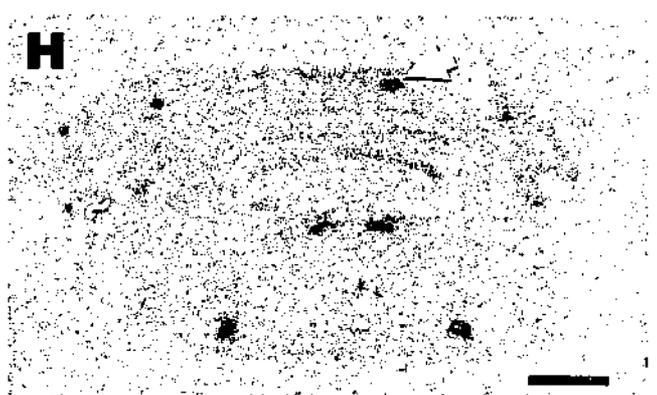
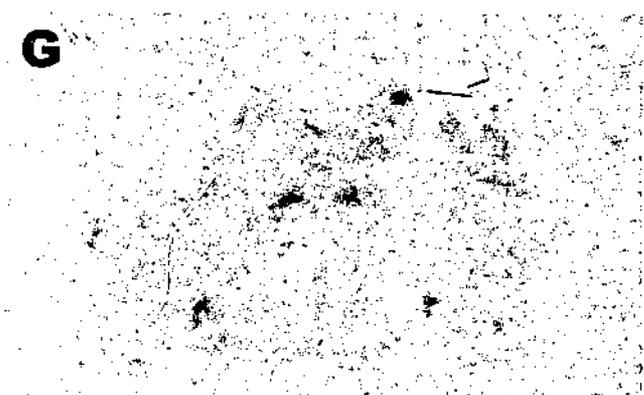
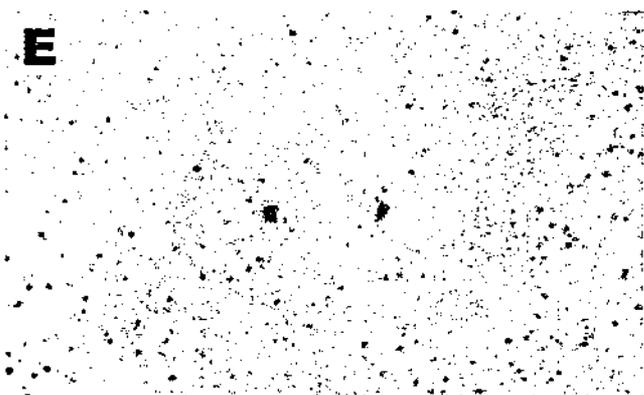
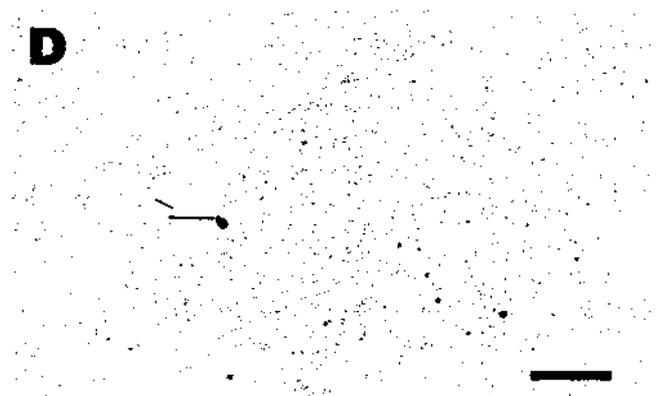
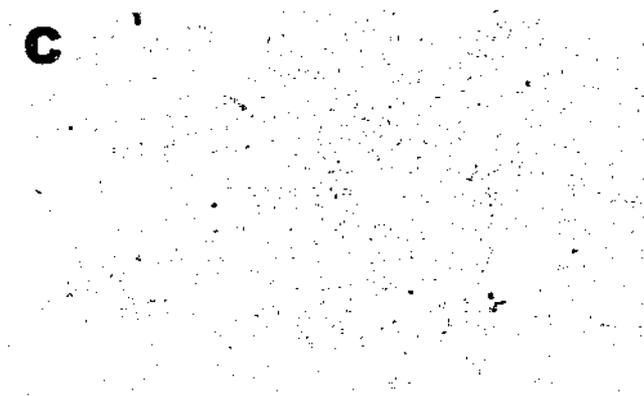
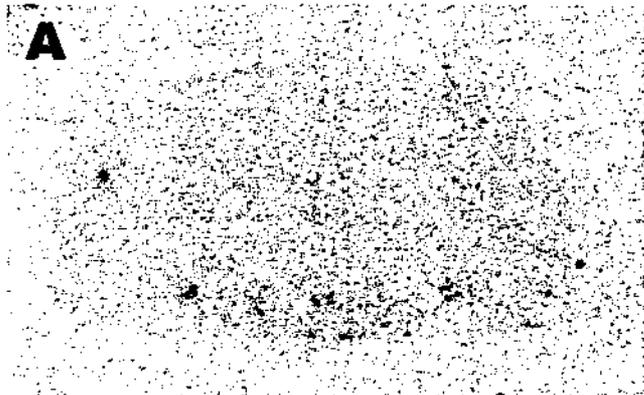
**FIGURE 3.13**

High power dark-field (A, C, E, G, I, K) and corresponding light-field (B, D, F, H, J, L) photomicrographs of emulsion-dipped slides demonstrating the presence of perikarya expressing prepro-GAL mRNA in various brain nuclei (A, B – Ce; C, D – PVN; E, F – DM; G, H – SON; I-L – LC). Silver grains represent the presence of prepro-GAL mRNA. Note the absence of prepro-GAL mRNA in cells bordering the third ventricle (3V) in panels C and D. Scale bar represents 200 $\mu$ m (A, B, C, D, G, H, K, L) or 100 $\mu$ m (E, F, I, J). For abbreviations, see page xvii.



**FIGURE 3.14**

Autoradiographic images of prepro-GAL mRNA hybridisation in the forebrain (A-D), pons (E, F) and medulla oblongata (G, H) of normotensive (WKY; A, C, E, G) and hypertensive (SHR; B, D, F, H) rat brain. Panels C and D represent the non-specific hybridisation of prepro-GAL in the forebrain of WKY and SHR respectively, which is indicative of the extremely low level of non-specific hybridisation observed throughout the CNS of both strains. White arrows point to random artifacts resulting from the ISHH procedure. Scale bar represents 2.42mm (A-D), 2.28mm (E, F) or 1.33mm (G, H).



**FIGURE 3.15**

Comparison of basal (no stress) prepro-GAL mRNA hybridisation density in the CNS of normotensive (WKY) and hypertensive (SHR) rat strains (n=3 per strain). Density of prepro-GAL mRNA hybridisation is expressed as the % change in SHR compared to WKY. For abbreviations, see page xvii.

\*:  $P < 0.05$ ; unpaired student's *t*-test.

# % CHANGE IN SHR COMPARED TO WKY

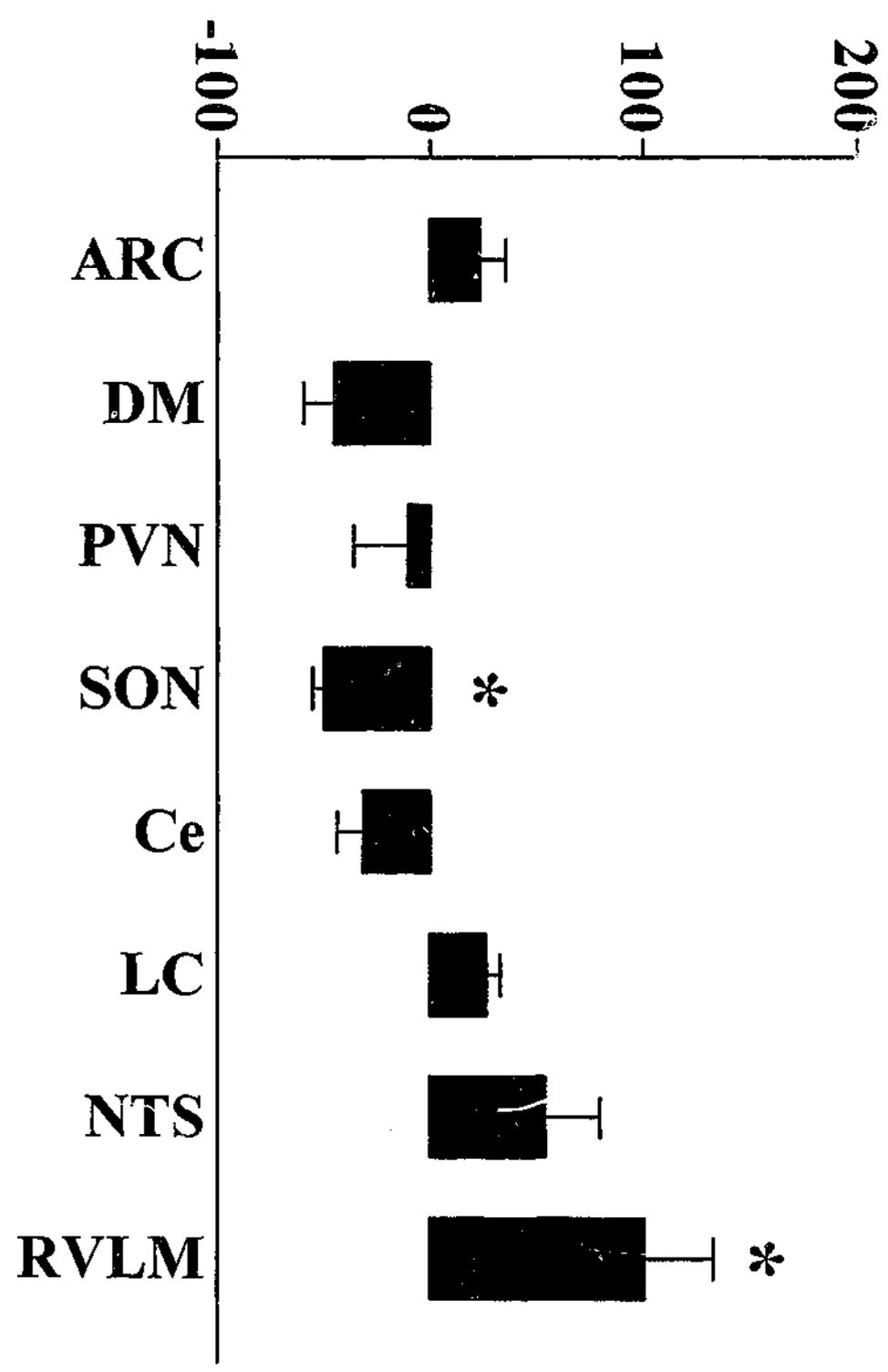


TABLE 3.3

REGION	RELATIVE DENSITY OF prepro-GAL mRNA	
	WKY	SHR
<b>FOREBRAIN</b>		
ARC	+	+
Ce	++	+
DM	++	+
LHy	+	+
PVN	+	+
SON	+	+
Pe	+	+
<b>PONS</b>		
LC	+++	+++
Cb	+	+
<b>MEDULLA</b>		
med NTS	+	++
RVLM	+	++

Relative distribution of prepro-GAL mRNA in the CNS of WKY and SHR rats. The density of prepro-GAL mRNA hybridisation was assessed as follows: +++ - dense ( $> 5$  DPM/mm<sup>2</sup>); ++ - moderate (2.5 DPM/mm<sup>2</sup> to 5 DPM/mm<sup>2</sup>) and + - light ( $< 2.5$  DPM/mm<sup>2</sup>). For abbreviations, see page xvii.

#### 3.3.4.2 Restraint stress and prepro-GAL mRNA expression

Exposure to restraint stress elicited alterations in prepro-GAL mRNA expression in a number of central regions in WKY and SHR (Figure 3.16). Compared to unstressed controls, a significant increase in the expression of prepro-GAL mRNA of +135% was observed after 1 session of restraint stress in the Ce of WKY ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test), and these levels returned to basal values on subsequent days (Figure 3.16A). In SHR, a significantly different temporal response to the restraint paradigm was detected in the Ce when compared to WKY ( $F(4,20) = 5.93$ ;  $P < 0.001$ ; two way ANOVA). While acute (1 period)

**FIGURE 3.16**

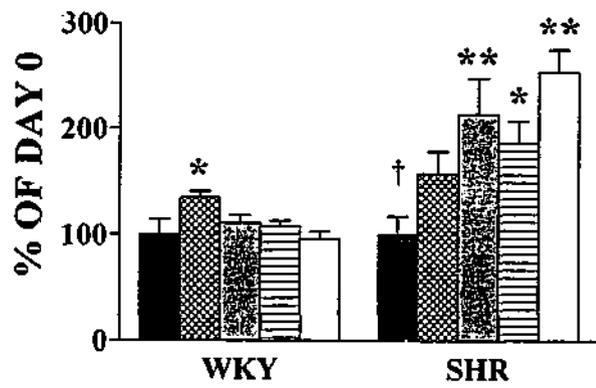
The effect of acute and chronic restraint stress on the density of prepro-GAL mRNA hybridisation in selected regions of normotensive (WKY) and hypertensive (SHR) rat CNS. Rats were exposed to a 60 min period of restraint stress for 0 (control; filled black columns), 1 (hatched columns), 3 (filled grey columns), 5 (horizontal lined columns) or 10 (open columns) consecutive days (n=3 rats per group per strain). Brain regions represented are: A – Ce; B – PVN; C – SON; D – LC; E – NTS and F – RVLM. Results are expressed as a percentage of day 0 (control) levels  $\pm$  S.E.M. For abbreviations, refer to page xvii.

\*:  $P < 0.05$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

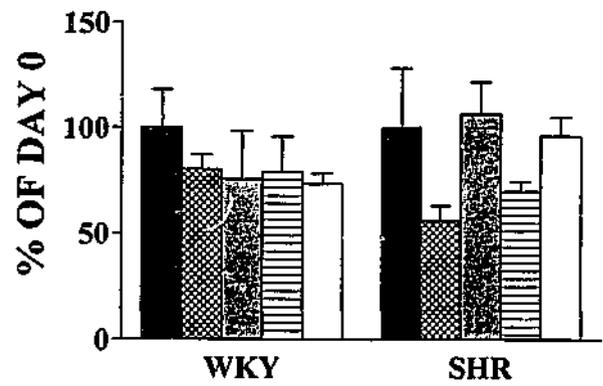
\*\* :  $P < 0.01$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

†:  $P < 0.05$  when comparing the temporal response to restraint in SHR with WKY; two way ANOVA.

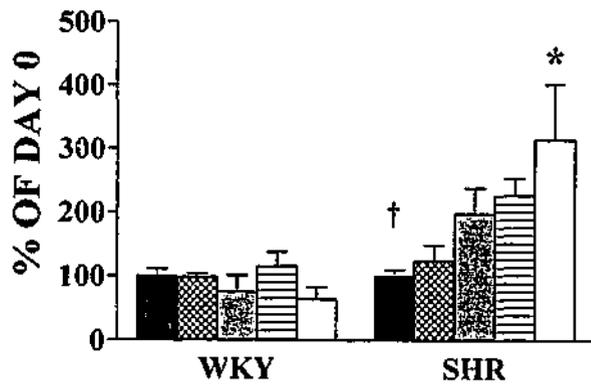
### A - Ce



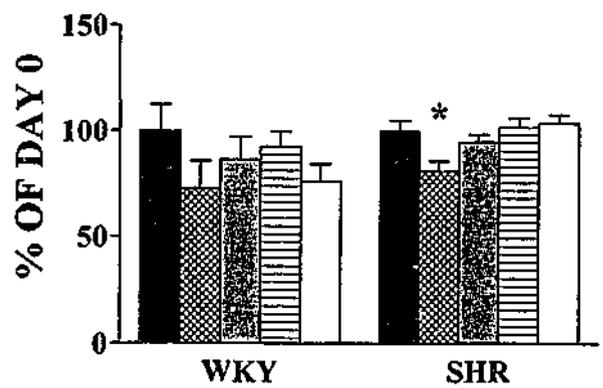
### B - PVN



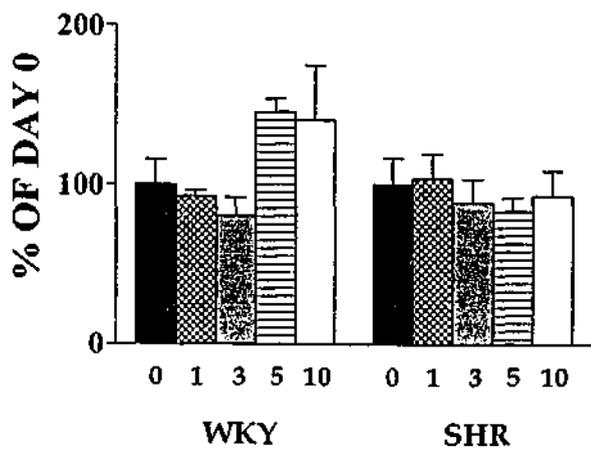
### C - SON



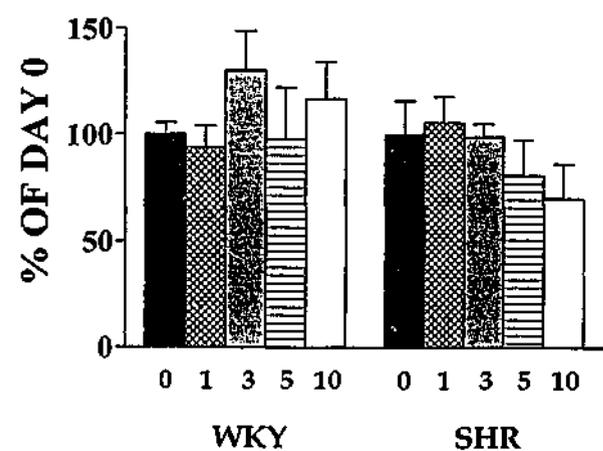
### D - LC



### E - NTS



### F - RVLM



restraint did not produce any significant effect on prepro-GAL mRNA expression in the Ce of SHR, 3, 5 and 10 periods of restraint elicited significant increases of up to +250% ( $P < 0.05$  (5 periods) and  $P < 0.01$  (3 and 10 periods); one way ANOVA with post-hoc Dunnett's test). This marked increase in prepro-GAL mRNA levels was observed after 10 sessions, and the pattern of prepro-GAL mRNA expression over the 10 day restraint paradigm clearly demonstrates that increased exposure to restraint produced a parallel increase in prepro-GAL mRNA levels in the Ce. Furthermore, at no time during the restraint paradigm did prepro-GAL mRNA expression in the Ce of SHR appear to decrease towards baseline levels of expression.

In SHR, while 1, 3 and 5 sessions of restraint did not elicit any significant changes in expression of the prepro-GAL transcript in the SON, a steadily increasing trend in prepro-GAL mRNA levels as the number of restraint sessions increased was apparent (Figure 3.16C). On the tenth day of the restraint protocol, a significant increase of  $\sim +300\%$  was observed ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test). In WKY, prepro-GAL mRNA expression did not increase or decrease in SON neurons in response to acute or chronic restraint. The markedly different temporal response observed in the SON of both strains was supported by the results of a two way ANOVA, which revealed a significant interaction between strain and the restraint paradigm ( $F(4,20) = 3.09$ ;  $P < 0.05$ ; two way ANOVA).

Prepro-GAL mRNA levels decreased significantly (-19%) in the SHR LC after 1 session of restraint ( $P < 0.05$ ; one way ANOVA), but demonstrated a steady return to basal levels as the number of stress sessions increased. In WKY, no significant changes in prepro-GAL mRNA levels were observed during the 10 sessions of restraint, although a reduction of -27% was observed after 1 period of restraint (Figure 3.16D). The response profiles in each strain were similar, an observation supported by the finding that there was no significant effect of strain on the response to restraint in the LC (two way ANOVA). No other significant restraint-induced changes were seen in the WKY or SHR CNS, although there was a trend towards an increase in prepro-GAL mRNA levels in the NTS of WKY following 10 consecutive days of the restraint paradigm (Figure 3.16E).

### 3.3.5 PREPRO-NPY mRNA EXPRESSION

#### 3.3.5.1 Strain comparison

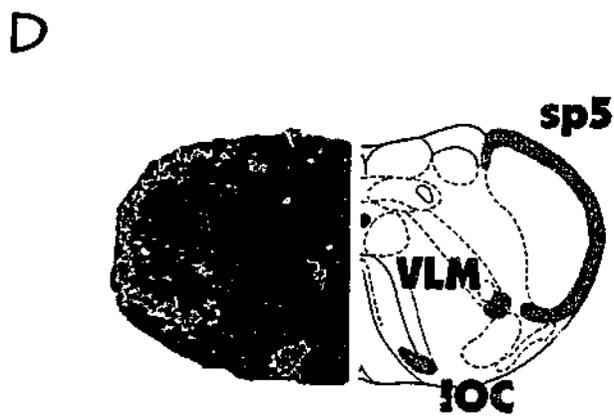
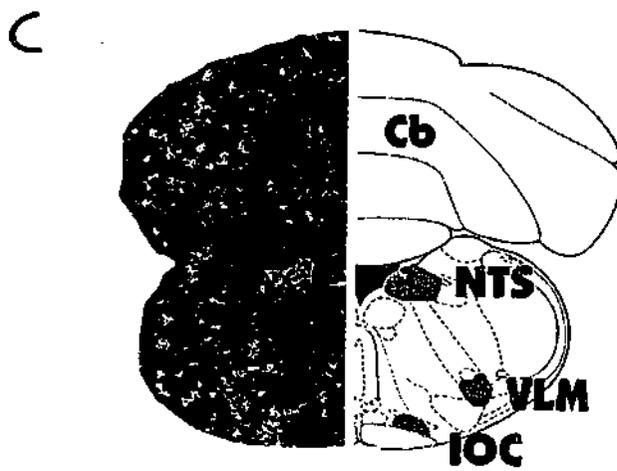
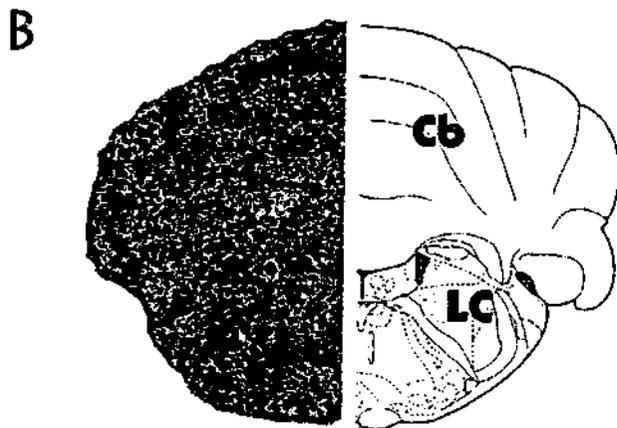
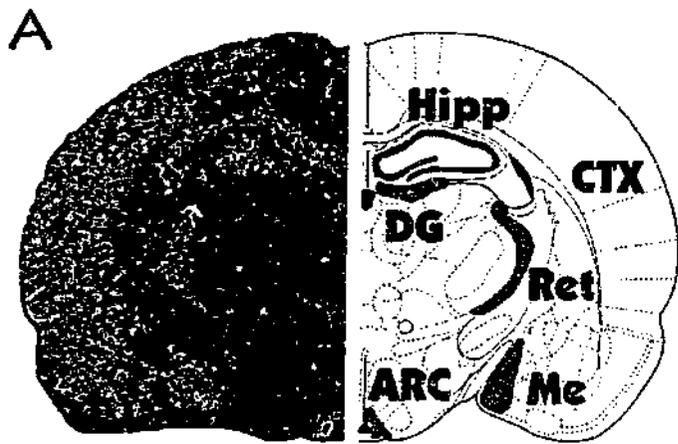
Prepro-NPY mRNA expression was detected in many regions throughout the CNS of both the normotensive and hypertensive strains (Table 3.4). In the forebrain, the prepro-NPY transcript was localised in the ARC, Me, hippocampus, DG, Ret and cortex (Figure 3.17). The expression of prepro-NPY mRNA was also quantified separately in the dorsal part of Ret (DRet), as the increased density of prepro-NPY mRNA observed in both WKY and SHR in this dorsal aspect facilitated delineation. The topographical distribution of prepro-NPY mRNA was difficult to attribute to a specific cortical subregion; thus, the cortex data are representative of all cortical regions at the level of the PVN, including the Par, Fr and Pir. A sparse distribution of prepro-NPY mRNA was also detected throughout the amygdala, although prepro-NPY mRNA was quantified only in the Me. Pontine and medullary nuclei containing detectable levels of prepro-NPY mRNA included the LC, NTS, VLM, sp5, IOC and cerebellum (Figure 3.17 and 3.18). Figure 3.18 demonstrates that a scattered pattern of prepro-NPY mRNA distribution exists in many nuclei such as the cortex and amygdala, suggesting that prepro-NPY mRNA is localised to neuronal clusters within these nuclei. In contrast, regions such as the ARC, LC and DG have a denser and more evenly distributed expression of prepro-NPY mRNA throughout the nucleus, indicating that a greater proportion of neurons within these nuclei contain prepro-NPY mRNA. The levels of prepro-NPY mRNA expression were quantified in all of these central nuclei, except for the cerebellum and in the amygdala where delineation of amygdaloid subnuclei other than the Me was difficult. The distribution of prepro-NPY mRNA was similar between WKY and SHR (Figure 3.18), and the basal level of expression of prepro-NPY mRNA was compared to determine the presence of any underlying differences in NPY neurochemistry in the hypertensive state. A statistical comparison using the unpaired student's *t*-test demonstrated that the level of prepro-NPY mRNA was significantly increased (+64%;  $P < 0.05$ ) in the ARC of SHR compared to WKY, with similar levels of expression found in the remaining nuclei (Figure 3.19).

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**FIGURE 3.17**

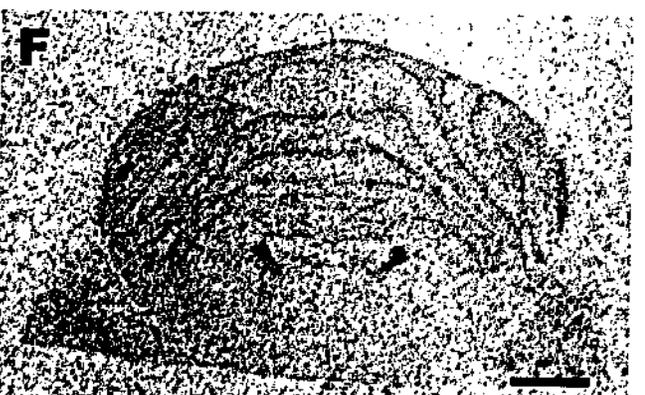
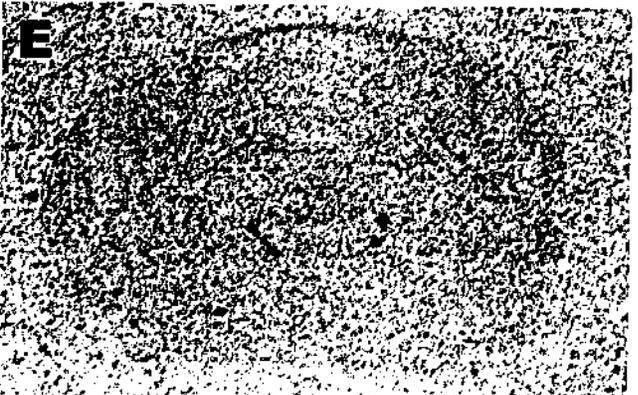
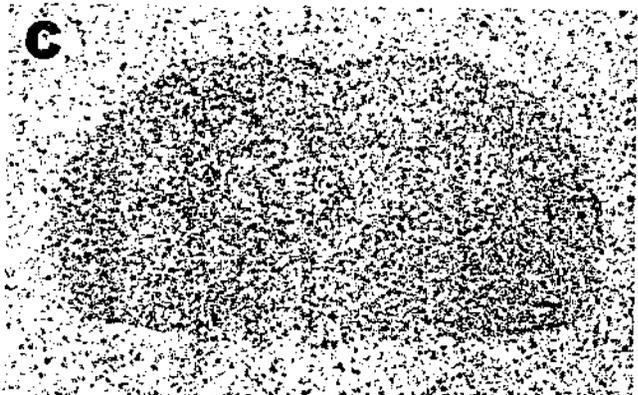
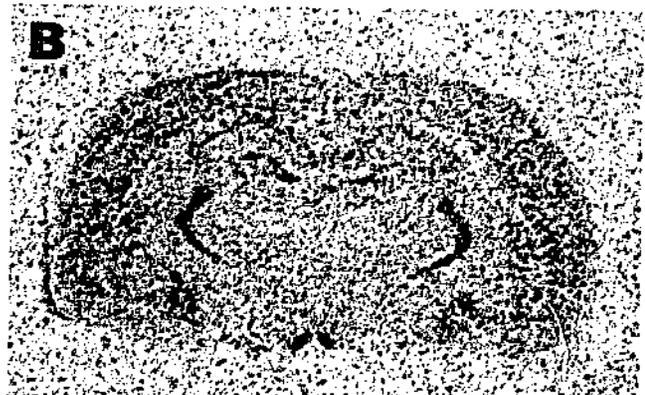
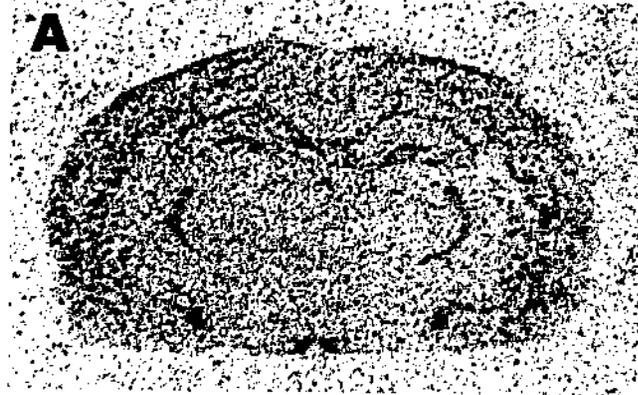
Representative pseudocolour autoradiograms and corresponding brain maps demonstrating the distribution of prepro-NPY mRNA hybridisation at four levels of the rat CNS (A – forebrain: bregma ~ -1.8mm; B – pons: bregma ~ -10.0mm; C – medulla oblongata: bregma ~ -13.3mm; D – medulla oblongata: bregma ~ -14.3mm). For abbreviations, refer to page xvii. Scale bar represents 1.88mm (A), 1.45mm (B), 1.37mm (C) and 1.1mm (D).

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**FIGURE 3.18**

Autoradiographic images of prepro-NPY mRNA hybridisation in the forebrain (A-D) and pons (E, F) of normotensive (WKY; A, C, E) and hypertensive (SHR; B, D, F) rat brain. Panels C and D represent the non-specific hybridisation of prepro-NPY in the forebrain of WKY and SHR respectively, which is indicative of the low level of non-specific hybridisation observed throughout the CNS of both strains. Scale bar represents 2.42mm (A-D) or 2.28mm (E, F).



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**FIGURE 3.19**

Comparison of basal (no stress) prepro-NPY mRNA hybridisation density in the CNS of normotensive (WKY) and hypertensive (SHR) rat strains (n=3 per strain). Density of prepro-NPY mRNA hybridisation is expressed as the % change in SHR compared to WKY. For abbreviations, see page xvii.

\*:  $P < 0.05$ ; unpaired student's *t*-test.

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# % CHANGE IN SHR COMPARED TO WKY

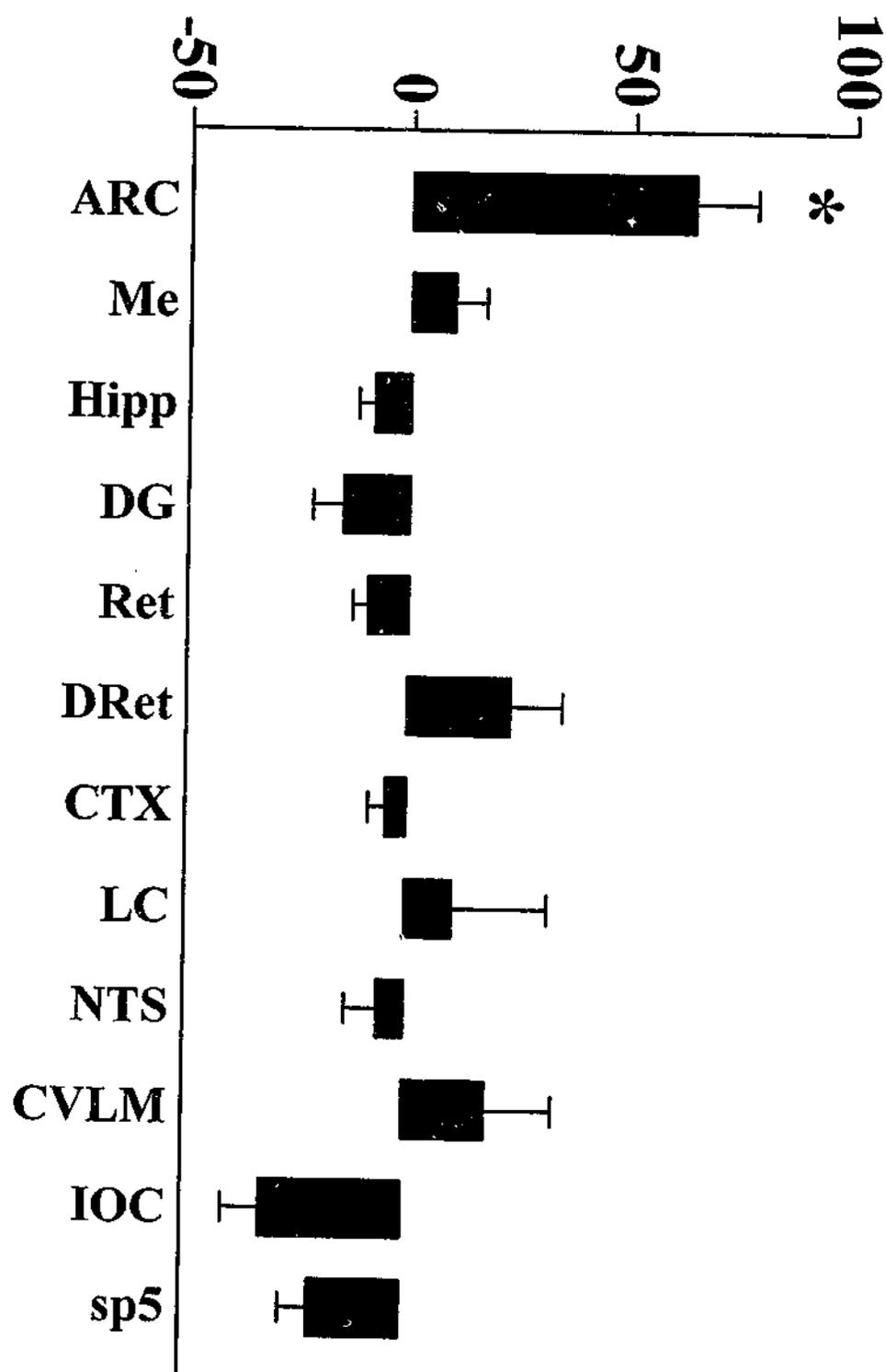


TABLE 3.4

REGION	RELATIVE DENSITY OF prepro-NPY mRNA	
	WKY	SHR
<b>FOREBRAIN</b>		
ARC	+++	+++
CPu	++	++
Me	++	++
Hipp	++	++
DG	++	++
Ret	++	++
DRet	++	++
CTX	++	++
<b>PONS</b>		
LC	+	+
Cb	+	+
<b>MEDULLA</b>		
NTS	+	+
IOC	+	+
sp5	+	+
VLM	+	+

Relative distribution of prepro-NPY mRNA in the CNS of WKY and SHR rats. The density of prepro-NPY mRNA hybridisation was assessed as follows: +++ - dense ( $> 2$  DPM/mm<sup>2</sup>); ++ - moderate (1 DPM/mm<sup>2</sup> to 2 DPM/mm<sup>2</sup>) and + - light ( $< 1$  DPM/mm<sup>2</sup>). For abbreviations, see page xvii.

### 3.3.5.2 Restraint stress and prepro-NPY mRNA expression

Restraint stress induced significant changes in the expression of prepro-NPY mRNA in the CNS of both strains (Figure 3.20). In WKY, a significant increase in prepro-NPY mRNA expression was detected in the ARC after 1 period of restraint stress (+81%;  $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test) (Figure 3.20A). Exposure to 3, 5 and 10 days of the

**FIGURE 3.20**

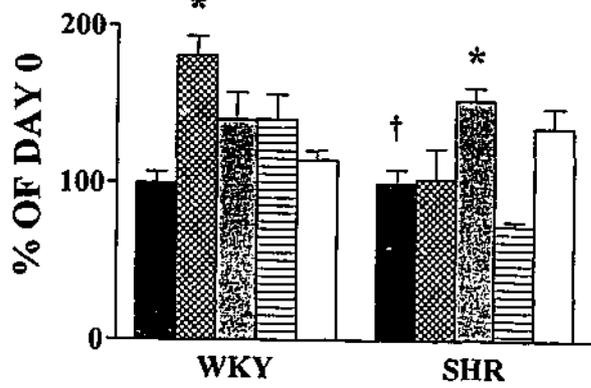
The effect of acute and chronic restraint stress on the density of prepro-NPY mRNA hybridisation in selected regions of normotensive (WKY) and hypertensive (SHR) rat CNS. Rats were exposed to a 60 min period of restraint stress for 0 (control; filled black columns), 1 (hatched columns), 3 (filled grey columns), 5 (horizontal lined columns) or 10 (open columns) consecutive days (n=3 rats per group per strain). Brain regions represented are: A – ARC; B – DG; C – CTX; D – LC; E – NTS and F – VLM. Results are expressed as a percentage of day 0 (control) levels  $\pm$  S.E.M. For abbreviations, refer to page xvii.

\*:  $P < 0.05$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

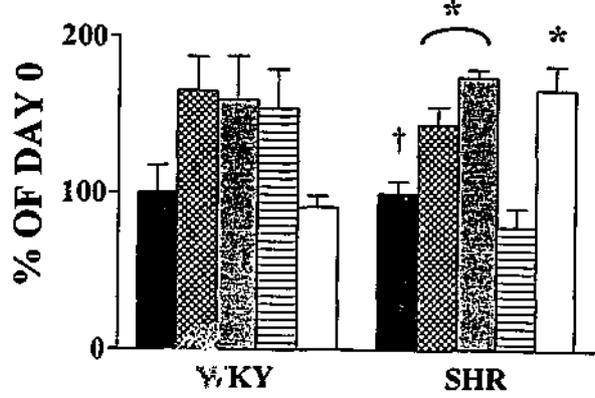
\*\* :  $P < 0.01$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

†:  $P < 0.05$  when comparing the temporal response to restraint in SHR with WKY; two way ANOVA.

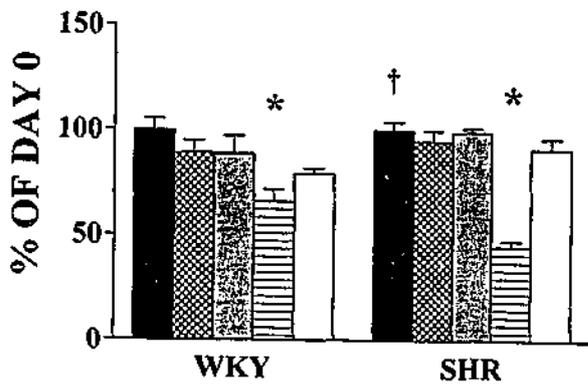
### A - ARC



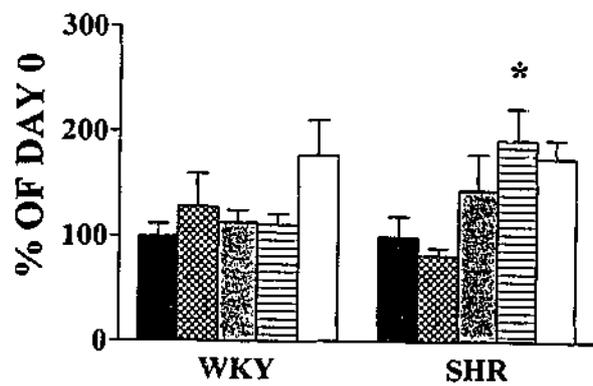
### B - DG



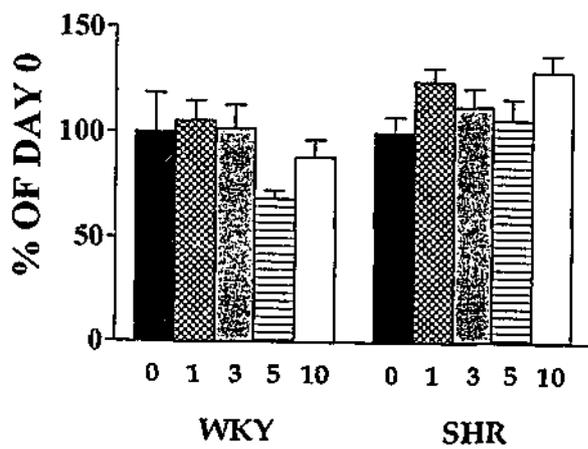
### C - CTX



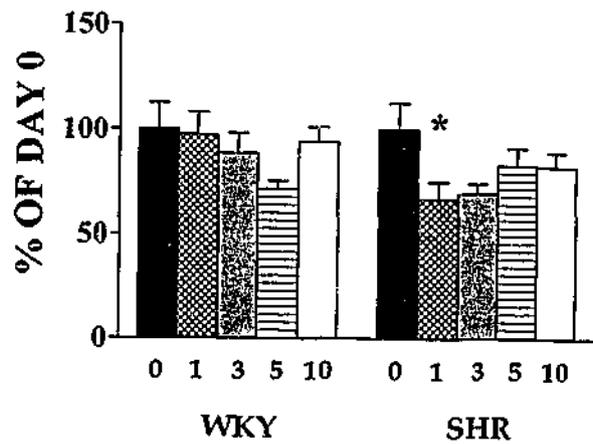
### D - LC



### E - NTS



### F - VLM



restraint paradigm did not produce the same significant elevation in prepro-NPY mRNA expression in the ARC of WKY. In the ARC of SHR, a significant elevation in levels of prepro-NPY mRNA was detected after 3 sessions of restraint (+53%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). This was followed by a rapid fall towards baseline levels of expression on the fifth day of the restraint paradigm. Comparison of the temporal stress response between WKY and SHR revealed the presence of a highly significant effect of strain on the response to restraint ( $F(4,20) = 7.45$ ;  $P < 0.001$ ; two way ANOVA).

Another region in the WKY CNS where the expression of prepro-NPY mRNA was significantly altered by restraint stress was the cortex (Figure 3.20C), where 5 periods of restraint resulted in a significant decrease of -34% ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test). A similar response was observed in the cortex of SHR after 5 sessions of restraint, although the decrease was slightly larger in magnitude (-55%;  $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test).

Significant increases in prepro-NPY mRNA expression were observed in the DG of SHR following 1 day (+44%;  $P < 0.05$ ), 3 days (+74%;  $P < 0.01$ ) and 10 days of the restraint paradigm (+67%;  $P < 0.01$ ) (one way ANOVA with post-hoc Dunnett's test). Despite the lack of any significant changes in the DG of WKY during the restraint paradigm, non-significant increases (~ +60%) in levels of the prepro-NPY transcript were observed in the DG of WKY after 1, 3 and 5 days of exposure to the restraint paradigm (Figure 3.20B). A significantly different temporal response to the restraint paradigm between strains was observed at the level of the DG ( $F(4,20) = 5.86$ ;  $P < 0.001$ ; two way ANOVA). Strain also exerted a significant influence on the response to acute and chronic restraint in the hippocampus ( $F(4,20) = 4.62$ ;  $P < 0.01$ ; two way ANOVA).

Further changes in prepro-NPY mRNA expression were detected in the pons and medulla of SHR. In contrast to forebrain nuclei such as the cortex, 5 periods of restraint resulted in a significant increase of +92% in the LC of SHR ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test) (Figure 3.20D). Neither acute nor chronic restraint elicited a significant change in prepro-NPY mRNA in any pontine or medullary region in the WKY strain. In contrast, many medullary regions in SHR exhibited restraint-induced changes in prepro-NPY mRNA levels. In the VLM of SHR, a significant decrease was observed after 1 session of restraint stress (-17%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test) (Figure 3.20F). The IOC in the ventral medulla in SHR exhibited significant increases in prepro-NPY mRNA levels following 5

sessions (+52%;  $P < 0.05$ ) and 10 sessions of restraint (+50%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). Finally, significant increases were also observed in sp5 of SHR following exposure to the restraint paradigm for 5 days (+53%;  $P < 0.01$ ) and 10 days (+44%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test).

As outlined above, exposure to restraint induced many more significant alterations in prepro-NPY mRNA expression in the CNS of SHR compared to WKY. Simple analysis of Figure 3.20 demonstrates marked differences in the temporal response pattern to restraint in a number of nuclei. In WKY, the majority of forebrain nuclei containing prepro-NPY mRNA were not perturbed by exposure to restraint stress. In contrast in the SHR strain, a biphasic response of prepro-NPY mRNA expression to restraint stress was observed in forebrain nuclei such as the DG, hippocampus, ARC, Ret, DRet and cortex. Specifically, an increase in prepro-NPY mRNA levels was often observed on the first and third day of the restraint paradigm. On the fifth day of the restraint paradigm, a rapid decline in prepro-NPY mRNA expression to levels equal to or below basal levels of expression was observed. This decrease was often followed by an elevation in the expression of prepro-NPY mRNA after 10 periods of restraint (Figure 3.20A-C).

## 3.4 DISCUSSION

### 3.4.1 RESTRAINT STRESS CONSIDERATIONS

The restraint stress experiments were performed in a laboratory remote from the animal holding area and as a consequence, all rats were subjected to the same procedure of transfer from holding facility to laboratory. Rats were kept in their home cages at all times, and in an attempt to reduce the possible confounding effects of transport and a change in surroundings, all rats were allowed at least 30 min to acclimatise to the laboratory. Control rats were also subjected to this change of environment, and while this change of environment may not reflect a true "no stress" control, the restraint procedure was the only difference in protocol between the control and restrained rat groups. As such, any changes observed in the restrained rats compared to the control group are clearly in addition to any possible effect of environmental stress. As demonstrated by the region-, neuropeptide- and strain-specific alterations that were detected in

this chapter (and in chapter 4), exposure to restraint is a greater stress than transport and a novel environment.

### 3.4.2 PREPRO-ENK mRNA EXPRESSION

#### 3.4.2.1 Strain comparison of basal expression

The mRNA encoding the precursor for the enkephalins, prepro-ENK, was detected in sections in multiple brain regions in the forebrain, pons and medulla using a 45-mer oligonucleotide probe. Oligonucleotide probes targetted at prepro-ENK have been used successfully in previous ISHH studies, and a distribution profile of prepro-ENK mRNA consistent with these reports was observed (Harlan *et al.*, 1987; Cowen *et al.*, 1998). Forebrain regions such as the CPu, Ce, PVN, PeF and VMH contained a high density of prepro-ENK mRNA, with high levels of prepro-ENK mRNA also found in the tegmental area (Teg), RMag, cerebellum, NTS and medullary reticular fields.

While both the normotensive and hypertensive rat strains exhibited a similar distribution of prepro-ENK mRNA, closer comparison of the basal expression of prepro-ENK mRNA revealed that significant differences existed in regions such as the Tz, cerebellum and RVLM. In all of these nuclei, the levels of prepro-ENK mRNA were significantly elevated by up to +58% in the SHR strain compared to WKY. The Tz has not been studied in as much detail as many central nuclei. Studies have shown that it is an integral part of central auditory pathways, with auditory stimuli such as white noise inducing the expression of *c-fos* mRNA in this region (Saint Marie *et al.*, 1999). However, strain differences between WKY and SHR relating to auditory performance have not been documented, and as a result it is unclear what effect altered prepro-ENK mRNA expression in the Tz has, if any, on the phenotype of the SHR. Further studies into the role of the Tz in hearing and other functions may provide a better understanding of the influence of the opioids in SHR in this region.

In the present study, the use of ISHH demonstrated that prepro-ENK mRNA was clearly confined to the granular cell layer of the cerebellum of both strains. Subjectively, there appeared to be no discernible difference in prepro-ENK mRNA content between the cerebellar layers and levels in either strain, and as such, the levels of prepro-ENK mRNA were presented for the cerebellum as a whole. The finding that prepro-ENK mRNA is significantly increased in the

cerebellum of SHR compared to WKY is in agreement with the results of Hoegler and co-workers who also reported an increase of  $\sim +66\%$  (Hoegler *et al.*, 1989). The cerebellum has been implicated in the modulation of HR and BP (Talman *et al.*, 1991), with spinally-projecting enkephalin-containing neurons located in the fastigial nucleus (Ikeda *et al.*, 1995), suggesting that these differences in prepro-ENK gene expression may be associated with the hypertensive state that is characteristic of the SHR strain. Studies have also shown that SHR are hyperactive compared to normotensive controls (Sagvolden *et al.*, 1993). Thus, evidence supporting a role for the cerebellum in the control of movement suggests that altered expression of prepro-ENK mRNA may contribute to this behavioural difference (Cicirata *et al.*, 1989).

The RVLM is the third relay nucleus in the central sympathetic loop that has a pivotal role in the control of cardiovascular homeostasis (Dampney, 1994). Thus, any neurochemical alterations present in the RVLM may exert an influence on the cardiovascular system. In the present study, prepro-ENK mRNA was found to be significantly increased in the RVLM of SHR compared to WKY. In hypertension, evidence exists demonstrating that the inhibitory influence of CVLM neurons is reduced, leading to a concomitant increase in the firing of barosensitive neurons in the RVLM (Chalmers *et al.*, 1992). Therefore, a release from the CVLM-mediated inhibition during hypertension may also produce an elevation in the levels of prepro-ENK mRNA in RVLM efferent neurons. Regions that receive projections from the RVLM that contain enkephalin, such as the LC and spinal cord, may also be affected by an altered expression of prepro-ENK mRNA in RVLM neurons (Menetrey & Basbaum, 1987; Drolet *et al.*, 1992). In addition, it has been shown that the non-C1 vasomotor neurons of the RVLM that project to the spinal cord contain prepro-ENK mRNA, suggesting that increased prepro-ENK mRNA in the RVLM of SHR may be contributing to elevated BP status (Guyenet *et al.*, 2001).

The present results are in contrast to the findings of Boone and colleagues (Boone & McMillen, 1994b), where ISHH was used to compare the expression of prepro-ENK mRNA in WKY and SHR in regions integral to cardiovascular control. SHR were found to have a reduced expression of prepro-ENK mRNA in the RVLM, CVLM and NTS, and these changes were postulated to contribute to the altered baroreceptor reflex in SHR (Boone & McMillen, 1994b). However, *all* of the reductions in prepro-ENK mRNA observed in the NTS, CVLM and RVLM by Boone and colleagues cannot solely affect baroreceptor reflex function because of the relationship between the NTS, CVLM and RVLM (Boone & McMillen, 1994b). The connections between the NTS and CVLM and between the RVLM and intermediolateral cell column (IML) are known to be stimulatory, while an inhibitory connection exists between the

CVLM and RVLM. Furthermore, microinjection of the mixed  $\mu/\delta$ -opioid receptor agonist DADLE and the  $\mu$ -opioid receptor agonist DAGO into the RVLM induced a hypotension, while a pressor effect was observed after microinjection of D-Ala<sup>2</sup>-Met<sup>5</sup>-enkephalinamide (DAME) into the CVLM (Willette *et al.*, 1984b; Sun *et al.*, 1996). Therefore, it would be expected that at least one of the regions would exhibit a change in prepro-ENK mRNA expression that is opposite in direction to the others. As a result, the differences in SHR and WKY prepro-ENK mRNA levels detected in the study by Boone and co-workers may ultimately be involved in any one of the many phenotypical differences between SHR and WKY, of which hypertension and an altered baroreceptor reflex is only one (Boone & McMillen, 1994b).

The LC and hypothalamic subregions were also found to contain significantly increased levels of prepro-ENK mRNA in SHR compared to WKY (Boone & McMillen, 1994b). In contrast, the present study did not report any significant changes in prepro-ENK mRNA in these regions. However, non-significant increases in prepro-ENK transcript levels were detected in the LC and PeF respectively in SHR compared to WKY. Thus, the results of the study by Boone and colleagues are indeed similar to the findings of the present study, and the large standard errors associated with the present results suggest that an increase in subject number may provide a clearer indication of whether these differences in prepro-ENK mRNA levels are significant (Boone & McMillen, 1994b).

In the rat, the PeF has been shown to send efferents to many regions, including the pPVN and mPVN, lateral septum, hippocampus, cortex (insular, medial prefrontal, Pir), PAG, PB, Barrington's nucleus, NTS and IML (Saper, 1985; Moga *et al.*, 1990b; Merchenthaler, 1991; Allen & Cechetto, 1992; Larsen *et al.*, 1994a; Valentino *et al.*, 1994). As a consequence of these connections, the PeF can have a direct modulatory influence on cardiovascular homeostasis through regions such as the NTS and IML. Furthermore, the efferent input to the pPVN may also be important in controlling the status of the sympathetic nervous system, as intra-PVN microinjection of  $\mu$ -opioid receptor agonists such as DAGO have been shown to increase BP and HR and produce elevated plasma catecholamine concentrations in conscious rats (Kiritsy-Roy *et al.*, 1986; Bachelard & Pitre, 1995). This hypothalamic region, therefore, may represent a novel target for future experiments investigating central nuclei that may contribute to increased sympathetic outflow and elevated blood pressure status in SHR.

### 3.4.2.2 Effects of restraint stress

Rats from both WKY and SHR strains were exposed to restraint, a robust psychological stressor (see section 1.2), for 60 min on 1, 3, 5 or 10 consecutive days and were subsequently processed for prepro-ENK mRNA using ISHH. Many regions within the CNS of both strains contained neurons where prepro-ENK mRNA synthesis was sensitive to perturbation by acute and/or chronic restraint stress. Previously published data support these findings, where psychological stressors such as restraint, as well as physical stressors, have been shown to induce changes in the central expression of prepro-ENK mRNA (Iglesias *et al.*, 1992; Young & Lightman, 1992; Ceccatelli & Orazzo, 1993; Boone & McMillen, 1994a; Larsen & Mau, 1994).

The present study found that acute restraint increased prepro-ENK mRNA in the PeF of WKY by +126%. Further exposure to restraint produced a graded return to baseline levels of prepro-ENK mRNA, indicating that either these neurons or the afferent input to these PeF neurons were adapting their activity in response to chronic restraint. In contrast, no changes in prepro-ENK mRNA expression were observed at any time in the PeF of SHR, demonstrating the difference in the temporal response to restraint between SHR and WKY at the level of the PeF. This is not the first report of an involvement of the PeF in the stress response, with psychological stressors such as immobilisation and conditioned fear and physical stressors such as water deprivation, haemorrhage and cold stress activating the neurons within the PeF region (Grinevich *et al.*, 1993; Fénelon *et al.*, 1994; Carrive, 1998).

The PeF receives afferent input from the amygdala (Ce, BL and Me) (Cassell *et al.*, 1986; Sakanaka *et al.*, 1986), and as described in the previous section, the PeF projects to many regions such as the pPVN, mPVN, Ce, supramammillary nucleus, thalamus, cortex (medial prefrontal, Pir, retrosplenial (RS)), hippocampus, NTS and IML (Swanson & Kuypers, 1980; Van der Kooy *et al.*, 1984; Saper, 1985; Cechetto & Saper, 1988; Larsen *et al.*, 1994a). The PeF may therefore serve as a relay point between regions involved in emotion and stressor perception and the output pathways for the cardiovascular, neuroendocrine and sympathetic nervous systems. Le Doux and colleagues support this hypothesis, as they have demonstrated that chemical lesion of the PeF attenuates the pressor component of the conditioned fear response in the rat (LeDoux *et al.*, 1988). Moreover, they suggest that the PeF is acting downstream from the amygdala (Me and Ce), as the behavioural response to conditioned fear was not affected by PeF lesion, while Ce lesion abolished both components of the fear response. Further evidence for a role for the PeF in the cardiovascular response to stress was provided by the studies of Diamant (Diamant *et al.*,

1992) and Sakananka (Sakanaka *et al.*, 1986). Sakanaka and colleagues firstly identified a projection from the Ce to the PeF that contained CRF-ir (Sakanaka *et al.*, 1986), and the functional study of Diamant and co-workers demonstrated that microinjection of CRF in the PeF region of conscious rats produced a tachycardia and slight pressor response (Diamant *et al.*, 1992). In conjunction, these two studies suggest that the release of CRF in PeF from terminals of neurons originating in the Ce have the capacity to modulate BP and HR.

Data suggest that the PVN may be a central region that mediates the cardiovascular effects produced by stimulation of the PeF. As described in section 3.4.2.1, the PeF projects to the pPVN and microinjection of the  $\mu$ -opioid agonist DAGO into the PVN has been shown to produce an increase in BP, HR and plasma catecholamines in unstressed rats (Kiritsy-Roy *et al.*, 1986; Bachelard & Pitre, 1995); however, intra-PVN naloxone had no effect on plasma catecholamine levels or on HR or BP (Kiritsy-Roy *et al.*, 1986). Kiritsy-Roy and colleagues then proceeded to look at how opioids modulate cardiovascular and sympathetic nervous system indices during restraint at the level of the PVN. They found that intra-PVN naloxone augmented the restraint-induced release of adrenaline, but had no effect on NAdr plasma levels, HR or BP when compared to the "normal" stress response in intra-PVN saline-treated rats (Kiritsy-Roy *et al.*, 1986). Moreover, intra-PVN administration of DAGO in rats exposed to restraint attenuated the restraint-induced tachycardia, but had no effect on catecholamine release or BP. However, DAGO and naloxone were administered almost 40 min prior to the restraint paradigm, leaving some doubt as to whether the same effects would be observed if these opioid receptor ligands were administered immediately prior/after initiation of the restraint period. Despite this shortcoming, the results published by Kiritsy-Roy and colleagues suggest that the opioid system exerts a complex modulatory influence on the cardiovascular system and sympathetic outflow in the PVN during stress.

It is currently unknown whether the PeF contributes to the opioid-mediated modulation of HR, BP and plasma catecholamines at the level of the PVN. Moreover, the direct projection of the PeF to the brainstem and IML indicates that the PeF has the potential to exert direct effects on these physiological systems. The results of the present thesis clearly show that acute restraint only elicits an increase in prepro-ENK mRNA in the PeF of WKY, but not SHR. Therefore, if the PeF is involved in mediating the cardiovascular component of the stress response, then it may also contribute to the altered tachycardic and pressor response to restraint previously observed in SHR (McDougall *et al.*, 2000).

Careful experiments are required to determine how the PeF is involved in these components of the physiological stress response. One approach is to expose rats to a restraint paradigm and then inject a retrograde tracer such as fluoro-gold into the IML or PVN. The brains of these rats could then be processed for Fos immunohistochemistry and the number of cells containing both Fos protein and the tracer could be quantified in the PeF, PVN and other regions. This study would provide an indication of the level of involvement of PeF neurons in the response to restraint and what regions those activated neurons project to. In addition, this study could be combined with BP or HR measurement, or even a quantification of plasma hormones or catecholamines. A comparison between hypertensive and normotensive rats in these experiments would also contribute to our understanding of the altered stress response in the SHR strain.

In the VMH of WKY, prepro-ENK mRNA expression was not altered by acute or chronic restraint stress, complimenting the results of Cullinan and colleagues who demonstrated that neither restraint nor swim stress activated VMH neurons (Cullinan *et al.*, 1995). In contrast, enkephalin-containing neurons in the VMH of SHR appeared to have an increased sensitivity to the restraint paradigm, with chronic periods of restraint significantly increasing prepro-ENK mRNA levels in this region. Considering that SHR also exhibit an altered cardiovascular response to stress (Li *et al.*, 1997; McDougall *et al.*, 2000), it is interesting to note that the VMH may be associated with the emotional stress-induced pressor response mediated by the Ce (Wu *et al.*, 1999). In this study, Wu and colleagues microinjected a CRF antagonist ( $\alpha$ -helical CRF) into the VMH and found that it had no effects on resting BP or HR, while microinjection of glutamate into the Ce produced a pressor response. When these two experiments were combined, such that  $\alpha$ -helical CRF was microinjected into the VMH 10 min before glutamate was microinjected into the Ce, the pressor response to intra-Ce glutamate was attenuated. The results of the study by Woo and co-workers (Wu *et al.*, 1999) share some parallels with the findings of Diamant (Diamant *et al.*, 1992), who demonstrated cardiovascular effects of CRF in the PeF, a region that also receives CRF-containing afferent input from the Ce. However, neither of these studies have been extended to determine whether either, both or none of these pathways are utilised during the response to psychological or physical stressors. For further investigation, CRF or its antagonist,  $\alpha$ -helical CRF, could be microinjected into both the PeF and VMH and the effects on the stress-induced cardiovascular response could be observed. This additional experiment should be performed in SHR and WKY rats, as it must be remembered that the results of the present thesis clearly showed strain differences in the involvement of the

PeF and VMH in the stress response. Specifically, prepro-ENK mRNA-containing neurons in the PeF of WKY were sensitive to acute restraint, while those in SHR were not, and prepro-ENK mRNA expression in the VMH of WKY was not altered by restraint, while chronic restraint produced a significant increase in prepro-ENK mRNA levels in the SHR VMH. Moreover, alterations in prepro-ENK mRNA expression in the VMH during stress may also impact upon other physiological functions of SHR that are influenced by opioids at this level, including feeding, body temperature control and analgesia (Tepperman & Hirst, 1983; Yamano *et al.*, 1986). Future research in this area, such as the experiment described above, would provide more information regarding the circuitry governing the various aspects of the central stress response in both WKY and SHR.

In the present study, acute restraint produced a significant increase in prepro-ENK transcript levels in the CPu of WKY that was followed by a return to basal levels of expression after subsequent exposure to restraint. Thus, it appears that the stress-induced activation of enkephalin-containing neurons within the CPu of WKY depends on the number of previous exposures to the same stressor, with repeated exposure producing an adaptive response. In SHR, the CPu neurons containing prepro-ENK mRNA have a temporally different response to the restraint paradigm compared to WKY, with the first significant increase in prepro-ENK mRNA occurring after 3 periods of restraint. Moreover, chronic exposure to restraint also produced an elevated expression of prepro-ENK mRNA in the CPu of SHR, while in WKY chronic restraint had no effect on striatal prepro-ENK mRNA expression.

One study found that 2 and 3 hours of immobilisation stress followed by at least 13 hours recovery in the home cage had no effect on striatal prepro-ENK mRNA content, while a 16 hour continual exposure to the same stressor produced a significant decrease in prepro-ENK mRNA (Chao & McEwen, 1990). These findings suggest that length of the stressor and recovery time are important factors regulating prepro-ENK mRNA in the CPu, and the restraint paradigm differences between the study by Chao and colleagues (Chao & McEwen, 1990) and the present study may account for the discrepant results. In support of the findings of the present study, previous reports using *c-fos* mRNA as an indicator of cellular activation have demonstrated that stress can activate neurons within the CPu (Cullinan *et al.*, 1995; Emmert & Herman, 1999). Furthermore, the density of striatal  $\mu$ - and  $\delta$ -opioid receptors, as well as dopamine receptors were increased by immobilisation stress (Zeman *et al.*, 1988). It is interesting that dopamine receptors within the CPu are also sensitive to stress, as both the opioid and dopamine systems within the CPu share a complex modulatory relationship (Yonehara & Clouet, 1984; Angulo,

1992). Therefore, alterations in enkephalin neurotransmission may reflect concomitant changes in the stress-induced release of dopamine in the CPu and associated functions, including reward, analgesia, locomotor activity and olfaction (Yonehara & Clouet, 1984; Johnson & Stellar, 1994; Holmes, 1999). Extending these possibilities would provide targets for future research into the functional role of the striatal opioid system in the neural stress response.

In both strains, an increased expression of prepro-ENK mRNA was detected in the Ce during the restraint paradigm. The only variability between strains related to the onset of the response, with WKY rats responding within the first day of the restraint paradigm, while prepro-ENK mRNA was not increased in the Ce of SHR rats until day 3. Further analysis of the data suggest that the control of the enkephalin-containing neurons within the Ce of WKY, and to a lesser extent the SHR, is plastic during the restraint paradigm. While evidence such as this demonstrates that the Ce can be activated by psychological stressors such as restraint (Honkaniemi, 1992), other studies have not reported any changes in *c-fos* mRNA and hence neuronal activity in this amygdaloid nucleus following restraint (Chen & Herbert, 1995). The study of Chen and co-workers (Chen & Herbert, 1995) using *c-fos* mRNA ISHH was only able to measure increases in activity, not decreases, demonstrating that ISHH protocols targeted at neuropeptides that have a detectable basal level of expression provide a better indication of the response of a neuron to a particular stimulus.

In the Ce, the opioids and enkephalins have been shown to induce a variety of biological effects, ranging from attenuation of the formation of gastric ulcers induced by cold-restraint (Ray *et al.*, 1988), to the regulation of feeding (Giraudo *et al.*, 1998) and analgesia (Pavlovic *et al.*, 1996b). Further investigation of the pathways mediating the control of feeding and analgesia has produced some interesting results. The reciprocal projection between the Ce and the NTS has been regarded as important in feeding regulation. Although opioid agonists and antagonists, particularly those selective for the  $\mu$  subtype, can regulate feeding at either the Ce or NTS (Giraudo *et al.*, 1998), studies have shown that a direct enkephalin-containing projection from the Ce to the NTS does not exist (Veening *et al.*, 1984; Gray & Magnuson, 1987). Similarly, opioid antagonists such as naltrexone,  $\beta$ -funaltrexamine and naltrindole isothiocyanate microinjected into the PAG can attenuate the analgesia elicited by intra-Ce opioid administration of morphine and  $\beta$ -endorphin, but enkephalin-ir was not detected in the Ce efferent neurons that terminate in the PAG (Gray & Magnuson, 1992; Pavlovic *et al.*, 1996b). Thus, it appears that one or more additional nuclei may be acting as a relay between the Ce and NTS in feeding regulation and between the Ce and PAG in the modulation of analgesia. As demonstrated by the

functional microinjection studies of Pavlovic (Pavlovic *et al.*, 1996b) and Giraudo (Giraudo *et al.*, 1998), the afferent input into both the NTS and PAG contain opioids. Therefore, further tracing studies are required to investigate the neural network containing the Ce that regulates both feeding and analgesia. Moreover, if the Ce is an essential component of these regulatory pathways in the resting state, then additional experiments can be completed in rats exposed to restraint to assist in determining the effects of the restraint-induced elevated expression of prepro-ENK mRNA in the Ce on analgesia and feeding.

The Ce may have a role that assists in the coordination of the neuroendocrine and behavioural components of the central stress response. The majority of the enkephalin-containing neurons of the Ce contain glucocorticoid receptor-ir, providing a direct mechanism where corticosterone levels in the plasma can regulate expression of prepro-ENK mRNA (Honkaniemi *et al.*, 1992b). As described earlier, variable differences in the sensitivity or formation of the HPA axis response to restraint between WKY and SHR have been reported and further studies are required in this area. If a difference between strains was found to exist, then the co-existence of glucocorticoid receptor-ir and prepro-ENK mRNA in Ce neurons may partially account for the temporal differences in the response of enkephalin-containing Ce neurons to restraint observed in this study in WKY and SHR.

The pontine LC is a region that has been activated by a wide variety of physical and psychological stressors (Curtis *et al.*, 1993; Chen & Herbert, 1995; Cullinan *et al.*, 1995), and a recently published study suggests that the LC may be associated with the central perception of stress severity (Chowdhury *et al.*, 2000). With NAdr-containing projections emanating from the LC projecting to almost the entire neuraxis (see Foote *et al.*, 1983), the LC assumes an important regulatory role in the central stress response. The results of the present study, where prepro-ENK mRNA expression was altered in the LC of both WKY and SHR following restraint, therefore reinforces the role of the LC as an integral part of the neural response to psychological stressors. In the LC of WKY, the restraint paradigm produced a bell-shaped response curve, with prepro-ENK mRNA expression increasing on session 1 and 3 to reach a maximum of +148% and subsequently decreasing on session 5 and 10 to baseline levels of expression. In contrast, restraint-induced prepro-ENK mRNA expression in the LC of SHR exhibited a completely opposite temporal response to WKY. A bell-shaped response curve is also apparent, but a significant decrease of -59% was quantified after 3 periods of restraint. Similar to the WKY strain, 5 and 10 periods of restraint produced much smaller changes in prepro-ENK mRNA expression that were close to basal expression levels.

The markedly different stress responses observed in the LC of WKY and SHR are interesting, given that neurochemical differences at the level of the LC in SHR have been reported in previous studies (Engberg *et al.*, 1987; Boone & McMillen, 1994b; Conti *et al.*, 1997). Although a direct study of enkephalin-containing efferents of the LC has not been published, a projection containing enkephalin from the LC to sympathetic preganglionic neurons in the spinal cord has been found to exist (Romagnano *et al.*, 1991). This study, together with the ubiquitous innervation of the CNS by the LC, indicates that basal and stress-induced alterations in prepro-ENK mRNA may ultimately influence sympathetic outflow and many other functions, such as neuroendocrine activation, arousal and stress-induced behaviour (Butler *et al.*, 1990; Smagin *et al.*, 1996; Ziegler *et al.*, 1999). Increased or decreased expression of prepro-ENK mRNA during restraint may have effects on motor activity, with reports describing reductions in motor activity following opioid receptor stimulation in the LC (Yang *et al.*, 1998). Moreover, the altered enkephalin stress response observed in the LC of SHR may contribute to the increased reactivity to stress previously reported in SHR (Knardahl & Hendley, 1990; Li *et al.*, 1997).

Like the LC, the RVLM also sends efferent projections that contain prepro-ENK mRNA to the spinal cord (Menetrey & Basbaum, 1987; Guyenet *et al.*, 2001), and studies suggest that the enkephalins may be modulating analgesia, substance P release and sympathetic outflow at this level (Lembeck & Donnerer, 1985; Wong-Dusting & Rand, 1989; Calcagnetti *et al.*, 1992). Within the rat RVLM, opioids have been reported to attenuate respiration when administered directly into this region (Chen *et al.*, 1996b), and as respiratory and cardiovascular effects are closely correlated, it is not surprising that intra-RVLM  $\mu$ - and  $\delta$ -opioid agonists also attenuate HR and BP (Li *et al.*, 1995). Electrical stimulation of the tibial nerve results in muscle contraction, somatosympathetic A- and C-reflexes in the inferior cardiac nerve and tachycardia and hypertension (Li *et al.*, 1996b; Caringi *et al.*, 1998). Administration of opioid agonists into the RVLM was shown to enhance the C-reflex, as well as attenuate the hypertension and bradycardia induced by tibial nerve stimulation (Li *et al.*, 1996b; Caringi *et al.*, 1998). Indirect evidence also suggests that opioids may attenuate the pressor response that is often associated with noxious stimulation (Sun & Spyer, 1991). Thus, the enkephalins may have effects on a variety of components if released locally in the RVLM or from projection neurons during exposure to stress.

Reports of adaptation to chronic stress at the level of the RVLM have been published previously, with levels of enkephalin mRNA in the RVLM after 11 periods of immobilisation

found to be similar to resting concentrations (Mansi *et al.*, 2000). This study also found that 90 min of immobilisation caused an increase in the number of Fos positive/ENK mRNA positive cells in the RVLM. Although this finding is in contrast to the results of the present study, the immobilisation paradigm used in the study by Mansi and colleagues represents a larger stressor than the restraint paradigm of the present investigation (Mansi *et al.*, 2000). However, a non-significant increase in prepro-ENK mRNA in the RVLM of WKY after 1 period of restraint was observed in the present study, suggesting that a greater stress may induce a larger increase in prepro-ENK mRNA levels as observed by Mansi and colleagues (Mansi *et al.*, 2000).

The cerebellum in both strains was extremely sensitive to the restraint stress paradigm. In both WKY and SHR, 10 sessions of restraint significantly reduced prepro-ENK mRNA expression to approximately the same level. However, as basal levels of expression of prepro-ENK mRNA were significantly increased in SHR, the percentage reduction in levels of the prepro-ENK transcript in SHR was two-fold greater than WKY. One interesting facet of the temporal response of cerebellar enkephalin-containing neurons to restraint is that there appears to be no adaptation to chronic restraint. There have been few studies reporting changes in activation of cerebellar neurons by different stressors, with Cullinan and co-workers observing minor increases in *c-fos* mRNA levels following restraint, with more *c-fos* mRNA expressed after swim stress (Cullinan *et al.*, 1995). The granule cell layer of the cerebellum was the most sensitive to swim stress, and this is the same layer where prepro-ENK mRNA predominates. As described in earlier sections, *c-fos* ISHH cannot distinguish between cellular activity that is either reduced or unaltered following a stimulus. Thus, a lack of *c-fos* mRNA or Fos-ir in the cerebellum after various stimuli may represent either an inhibition or insensitivity to the stimulus. The present study therefore suggests that restraint and other stimuli may result in an inhibition of cerebellar neurons, particularly those containing prepro-ENK mRNA.

The enkephalin-containing neurons of the cerebellum have been shown to project to the spinal cord (Ikeda *et al.*, 1995), while other central projections of enkephalin-containing cerebellar neurons have not been detailed. The spinally projecting neurons have the ability to modulate spinal reflexes and motoneurons, as well as analgesia and the cardiovascular system. Supporting these hypotheses are data demonstrating that opioids within the cerebellum can produce analgesia (Dey & Ray, 1982), as well as evidence for a role for the cerebellum in cardiovascular and motor control (Cicirata *et al.*, 1989; Talman *et al.*, 1991). Further work is needed to determine whether restraint has the capacity to modify these functional effects through

the cerebellar enkephalin-containing neurons, and if so, whether these neurons project directly to the spinal cord or to a presently unknown central region.

It is interesting to note that the enkephalin-containing neurons of the LC, RVLM and cerebellum were all sensitive to perturbation by restraint stress. A common link between these three regions is apparent, with enkephalin-positive neurons from all regions projecting to various levels of the spinal cord. Further investigations would provide a clearer understanding of the role of these spinally projecting neurons and their involvement in the stress response. Research directions that may be addressed include a study of whether these three central regions are regulating a common target or function, such as sympathetic outflow or analgesia, and how these regions may be coordinating the behaviour (LC), cardiovascular (RVLM, together with the LC and cerebellum), motor (cerebellum) and neuroendocrine (LC) components of the stress response at the level of the spinal cord.

### 3.4.3 PRODYN mRNA EXPRESSION

#### 3.4.3.1 *Strain comparison of basal expression*

The distribution of neurons containing proDYN mRNA was investigated using a combination of 2 oligonucleotide probes. These probes were targetted at different portions of the proDYN mRNA sequence, and effectively increased the amplitude of the hybridisation signal. Previous studies of proDYN mRNA in the rat CNS have described a distribution of proDYN that was consistent with the results of the present study (Morris *et al.*, 1986; Mansour *et al.*, 1994b). The highest levels of proDYN mRNA were visualised in the PVN and SON, with moderate levels of expression of proDYN mRNA in other hypothalamic regions (DM and VMH), Ce, CPu, DG. In the pons and medulla, the distribution of proDYN mRNA was much more restricted, with only the NTS and ECu containing measurable levels of the proDYN transcript.

In brain sections obtained from both WKY and SHR, proDYN mRNA exhibited a similar distribution profile. Further comparison of the density of proDYN mRNA revealed that marked differences in basal levels of proDYN mRNA existed in some nuclei. In the SON, DG, CPu and NTS, proDYN mRNA levels were significantly lower in SHR compared to WKY, with the largest difference occurring in the DG (-73%). This finding is in agreement with earlier studies by Yin (Yin *et al.*, 1997) and Wang (Wang *et al.*, 1994b), who also reported a significantly

reduced expression of proDYN mRNA in the DG. Furthermore, levels of dynorphin A (1-8) were reduced in the hippocampus of SHR compared to WKY and SD at 4, 8 and 12 weeks of age (Li *et al.*, 1989; Privette *et al.*, 1994), while the density of dynorphin A (1-13) binding sites within the hippocampus was also found to be significantly lower in SHR than age-matched WKY (McConnaughey *et al.*, 1992). These data provide evidence that deficits in proDYN mRNA expression, peptide levels and receptor density may be associated with one or more of the phenotypic differences between WKY and SHR.

Opioid agonists selective for the  $\kappa$ -opioid receptor subtype such as dynorphin A (1-8) elicit hypotension and bradycardia when injected directly into the hippocampus of both normotensive and hypertensive rats (Wang & Ingenito, 1994a). However, dynorphin A (1-8) produced a larger hypotensive response in SHR rats compared to WKY, demonstrating that changes in proDYN mRNA levels and the concentration of opioid peptides that are obtained from proDYN such as dynorphin A (1-8) can be associated with concomitant changes in their particular receptor (Wang & Ingenito, 1994a). When the DG dynorphin-containing cell bodies of neurons that project to the hippocampus were destroyed by colchicine, a hypertensive response was observed in normotensive rats, while a hypotensive period lasting for 6 weeks was observed in SHR (Privette *et al.*, 1994). This evidence suggests that the dynorphin-containing neurons of the DG have the capacity to influence BP homeostasis, and that they produce quite different cardiovascular effects in WKY and SHR. A comparison of proDYN mRNA levels in additional strains of normotensive and hypertensive would subsequently demonstrate whether the differences observed in the present study are related to strain or the development/maintenance of hypertension.

The present study also found a significantly reduced expression of proDYN in the SON of SHR compared with normotensive WKY. However, there have been no direct investigations of proDYN mRNA expression in the SON of WKY and SHR, with a previous study that compared the basal levels of proDYN mRNA expression in the CNS of WKY and SHR failing to quantify the expression of proDYN mRNA in the SON. Indirect evidence that compliments the results of the present study have been provided by reports of significantly reduced dynorphin concentrations in the pituitary (Feuerstein *et al.*, 1983; Bhargava *et al.*, 1988), a region known to receive dynorphin-containing afferent input from the SON (Brown *et al.*, 2000). Contrasting results have been reported in studies measuring the levels of dynorphin-ir in whole hypothalami of SHR and normotensive rats, with significantly increased, reduced and unchanged levels of dynorphin-ir (Feuerstein *et al.*, 1983; Conway *et al.*, 1987; Bhargava *et al.*, 1988; Li *et al.*,

1992). These variable results are difficult to interpret, given that the present study and another study by Yin and colleagues (Yin *et al.*, 1997) reported *only* significantly reduced proDYN mRNA content in SHR hypothalamic subnuclei such as the SON, medial POA and VMH. Further investigations of these discrepancies and variability using immunohistochemical techniques with a much higher anatomical resolution may provide a clearer insight into the differences between hypertensive and normotensive rats relating to the hypothalamic dynorphin system.

The SON regulates the release of a number of hormones from the posterior pituitary, including vasopressin and oxytocin. Following peripheral (i.v.) administration of opioid agonists, such as morphine and the  $\kappa$ -opioid selective agonist U50-488H, the activity of oxytocin-containing neurons in the SON, and hence the release of oxytocin, was reduced (Ludwig *et al.*, 1997). Furthermore, there is indirect evidence indicating that intra-SON dynorphin elevated the release of vasopressin in anaesthetised rats, possibly through a non-opioid receptor as these effects were not blocked by naloxone (Tsushima *et al.*, 1993).

Oxytocin- and vasopressin-containing neurons of the SON have been shown to project to the neurohypophysis, and endogenous opioid peptides such as dynorphin and enkephalin have been colocalised in these neurons in the rat (Martin & Voigt, 1981; Molineaux *et al.*, 1984). Interestingly, adult SHR rats have elevated levels of vasopressin mRNA and reduced expression of the oxytocin transcript in SON neurons compared to normotensive controls (Van Tol *et al.*, 1988). Thus, it appears that in this hypertensive strain, neurochemical alterations in SON neurons depend on the neuropeptide. Further work is required to determine if the expression of proDYN mRNA is reduced in all SON neurons, if the significant reductions in proDYN mRNA are confined to the oxytocin-containing neurons and how these alterations affect neuronal activity during basal and stressful conditions.

Opioid receptors, primarily belonging to the  $\kappa$ -subtype, have been detected in the neurohypophysis (Herkenham *et al.*, 1986) and activation of these receptors inhibited the stimulated release of oxytocin and vasopressin (Zhao *et al.*, 1988). Therefore, alterations in the production of proDYN mRNA and its peptides may have detrimental effects on the release of oxytocin and vasopressin. Studies comparing plasma vasopressin levels between SHR and normotensive controls have revealed that hypertensive rats have augmented plasma vasopressin concentrations (Morris *et al.*, 1983; Rosella-Dampman *et al.*, 1985). Published comparisons of plasma oxytocin concentrations have produced contrasting results, with similar and reduced

plasma levels of oxytocin found in male SHR compared with WKY (Morris *et al.*, 1983; Rosella-Dampman *et al.*, 1985). These results compare favourably with the levels of vasopressin and oxytocin mRNA detected in the SON of SHR and WKY described above.

An investigation into the role of opioid-mediated modulation of the release of vasopressin demonstrated that naloxone elevated the plasma levels of vasopressin in SHR, but not in WKY or SD rats (Rosella-Dampman *et al.*, 1985). In addition, systemic naloxone had no effect on plasma oxytocin levels in SHR and SD, while in WKY, naloxone induced a significant increase in plasma oxytocin concentration. These findings demonstrate that the opioid-mediated modulation of vasopressin and oxytocin release is clearly different between WKY, SD and SHR, but these alterations may be related to strain, rather than the status of the cardiovascular system. The contribution of decreased proDYN mRNA expression in SHR to the regulation of the release of these hormones that is mediated by the opioid system would need to be investigated in more detail in future studies.

The CPu of SHR was found to contain a significantly lower level of proDYN mRNA when compared with WKY. Surprisingly, Yin and colleagues did not quantify proDYN mRNA levels in the striatum of WKY or SHR (Yin *et al.*, 1997). As such, the present study represents the first report of differences in proDYN mRNA expression in the CPu of SHR compared with normotensive controls. Once more, immunohistochemical studies of central dynorphin peptides in SHR and WKY produced conflicting results, with reports of significantly increased and decreased dynorphin concentrations in the CPu of SHR compared to WKY (Bhargava *et al.*, 1988; Tan-No *et al.*, 1997). The reason for the discrepancies between these studies is unclear. Tan-No and colleagues reported increased levels of both dynorphin A and dynorphin B in the CPu, while the study by Bhargava and co-workers reported decreased striatal dynorphin A (1-13) levels (Bhargava *et al.*, 1988; Tan-No *et al.*, 1997). Although both studies targeted different peptide fragments, it is hard to attribute the contrasting results to this protocol difference. Contributing factors to the discrepant results are more likely related to the slightly different ages of the rats in the study (8 weeks (Bhargava *et al.*, 1988) versus 10 weeks (Tan-No *et al.*, 1997)), and more importantly, the breeding stocks used for each rat strain. Notably, neither study obtained rats from the same source as the WKY and SHR rats used in the present study and it is possible that differences in dynorphin content of the CPu may be detected in these three different SHR and WKY colonies.

Dynorphin has been localised in the neurons intrinsic to the striato-nigral pathway, and as such, is in a position to regulate dopamine neurotransmission in both the CPU and substantia nigra (Fallon *et al.*, 1985; Reid *et al.*, 1990; Steiner & Gerfen, 1996). This close association between dynorphin and dopamine has provided the impetus for investigations of functions modulated by striatal dynorphin-containing neurons. Intrusion of spiradoline, a  $\kappa$ -opioid receptor agonist, into the striatum of normotensive rats attenuated the expression of *c-fos* mRNA in striatal neurons following systemic administration of the indirect dopamine  $D_1$  receptor agonist and CNS stimulant, cocaine (Steiner & Gerfen, 1995). Furthermore, microinjection of dynorphin and dynorphin fragments into the substantia nigra, a region that receives dynorphin-containing afferents from the striatum, was shown to induce circling in rats (Friederich *et al.*, 1987). In SHR, reduced expression of proDYN mRNA in the CPU may therefore influence dopamine transmission and associated functions such as reward and motor control. Interestingly, the electrically stimulated release of dopamine from striatal slices of SHR was found to be significantly lower than WKY (Tsuda *et al.*, 1998). In addition, inhibitors of dopamine neurotransmission such as GAL and NPY produce larger effects in striatal slices obtained from SHR than WKY, indirectly suggesting that the dynorphins may produce the same effect (Tsuda *et al.*, 1997; Tsuda *et al.*, 1998). Van den Buuse and colleagues investigated the effects of various dopamine agonists and antagonists on behaviour in SHR and WKY and reported that differences in the dopamine system exist between strains (Van den Buuse & de Jong, 1989). Further investigations of the striatal dopamine and dynorphin systems and correlations with behaviour may provide clearer insights into the interactions between the dopamine and opioid systems in the CNS of SHR and WKY.

Normotensive WKY and SHR were found to have comparable levels of proDYN mRNA in all other forebrain regions. However, the NTS was a medullary region where the level of proDYN mRNA was significantly lower in SHR than WKY. This result is in agreement with the study of Yin and co-workers (Yin *et al.*, 1997) who also found a reduction in the expression of proDYN mRNA in the NTS of SHR compared to normotensive controls. Immunohistochemical comparisons of dynorphin-ir in the NTS of normotensive and hypertensive rats have not been published, although there are studies that have compared dynorphin-ir in the pons and medulla as a whole (Bhargava *et al.*, 1988). In this particular study, Bhargava and colleagues found no difference in the content of pontine-medullary dynorphin-ir between WKY and SHR, but the size of the NTS compared with the pons and medulla means that any differences existing in the NTS would be considerably diluted using this analysis protocol (Bhargava *et al.*, 1988).

The neural connections of dynorphin-containing neurons in the NTS provide some indications of the potential causes and effects of reduced proDYN mRNA expression. Dynorphin-containing neurons within the medial and commissural NTS receive vagal and glossopharyngeal afferent input, and as such, receive information regarding the status of the cardiovascular system (Fodor *et al.*, 1990; Rutherford & Gundlach, 1993). These dynorphin-containing neurons are subsequently in a prime position to transmit vagal afferent signals to other central nuclei. Efferent projections from the NTS that contain dynorphin have been detected in the anterior hypothalamus, BNST and PB (Riche *et al.*, 1990). In the anterior hypothalamus, microinjection of dynorphin A (1-13) was associated with a hypotension and bradycardia (Rabkin, 1993), while another study demonstrated that microinjection of dynorphin A (1-17) in the anterior hypothalamus can induce a hypothermia (Xin *et al.*, 1997). Dynorphin-ir in the BNST and PB can be increased by food restriction, suggesting that  $\kappa$ -opioid receptors may be implicated in the modulation of appetite in these regions (Berman *et al.*, 1994). In addition, proDYN-containing neurons in the PB are involved in nociception (Hermanson *et al.*, 1998). Thus, changes in proDYN mRNA expression in the NTS of SHR may have effects on a variety of functions, including cardiovascular modulation.

Within the NTS itself,  $\kappa$ -opioid agonists such as dynorphin A (1-13), bremazocine and MRZ 2549 produce a hypotension and bradycardia, together with a decrease in tidal volume and respiratory rate in spontaneously breathing anaesthetised rats (Hassen *et al.*, 1984; Rabkin, 1993). Electrophysiological studies have demonstrated that activation of pre- and post-synaptic  $\kappa$ -opioid receptors within the NTS inhibited glutamate-induced excitatory postsynaptic potentials (EPSPs) and hyperpolarised several cells (Rhim *et al.*, 1993). Considering that glutamate is the primary transmitter in vagal afferents to the NTS (see Zhou *et al.*, 1997 and Lawrence & Jarrott, 1996), it is possible that activation of  $\kappa$ -opioid receptors may modulate vagal afferent transmission at the level of the NTS.

A role in the modulation of vagal afferent transmission may be important in hypertension, as a reduction in proDYN mRNA in intrinsic NTS neurons may result in a decreased inhibition of (glutamate) neurotransmission. Moreover, the studies of Hassen (Hassen *et al.*, 1984) and Rabkin (Rabkin, 1993) suggest that a reduction in proDYN mRNA in the NTS of SHR may contribute to the hypertensive state. The study published by Gordon, however, demonstrates that microinjection of naloxone into the NTS of normotensive rats has no effects on the central control of the baroreflex (Gordon, 1990). Thus, while dynorphin and other  $\kappa$ -opioid agonists have the capacity to modulate BP and HR at the level of the NTS, the opioid system does not

appear to be tonically involved in the central control of cardiovascular function. Hypertensive rats were not studied by Gordon (Gordon, 1990), and a subsequent study replicating the work of Gordon using SHR would demonstrate whether opioids have a tonic role in the regulation of HR and BP at the level of the NTS in hypertensive rats. In addition, further studies of the association between the dynorphin system in the NTS of SHR and hypertension are required, with a particular focus on immunohistochemical, autoradiographic and functional comparisons of  $\kappa$ -opioid receptors between strains.

#### 3.4.3.2 *Effects of restraint stress*

Following such a widespread involvement of central enkephalin neurons in the central response to acute and chronic restraint stress (section 3.3.2.2), it was of considerable interest to observe the sensitivity of another opioid system to the same stress paradigm. Furthermore, there is a paucity of studies investigating changes in the dynorphin system during exposure to stress, particularly in hypertensive rats. Using brain sections that were adjacent to those used in the prepro-ENK mRNA component of the present study, the expression of proDYN mRNA was quantified in WKY and SHR following exposure to 0 (control), 1, 3, 5 and 10 days of the restraint paradigm. Generally, dynorphin-containing neurons were sensitive to perturbation by both acute and chronic restraint, with region- and strain-specific changes observed mainly in the forebrain, but also in the NTS in hypertensive SHR.

ProDYN mRNA-containing neurons in the hypothalamic SON of SHR were found to respond differently to restraint stress when compared to WKY. In the SON of WKY, there were no significant changes in proDYN mRNA expression after any exposure to restraint. In contrast, acute and chronic restraint produced significant increases in proDYN mRNA levels in the SON of SHR of between +231% and +363%. The temporal response pattern also suggests that the dynorphin-containing neurons within the SON of SHR, and possibly their afferent input, were not adapting to the 10 day restraint paradigm. The dynorphin-containing neurons in the SON of SHR may therefore require more than 10 days of stress to adapt, or equally, they may be in a constantly activated state during exposure to stress. Further research using restraint or other stressors for more than 10 periods over 10 days may provide a better understanding of the activity of the dynorphin-containing neurons in the SON of SHR during chronic restraint.

Previous studies using psychological stressors such as restraint and swim stress have reported that Fos-ir or *c-fos* mRNA expression has increased in SON neurons of normotensive rats (Cullinan *et al.*, 1995; Krukoff & Khalili, 1997; Dayas *et al.*, 1999). Physical stressors such as i.p. administration of hypertonic saline have also been shown to consistently activate the neurons of the SON (Larsen & Mikkelsen, 1995). Comparative studies of neuronal activation in the SON of hypertensive rats following exposure to either psychological or physical stressors have been limited. Furthermore, there have been no previous comparisons of the response to stress exhibited by SON neurons of WKY and SHR containing dynorphin, whether it be studies quantifying changes in proDYN mRNA, dynorphin-ir or density of the  $\kappa$ -opioid receptor. As such, the present study represents the first investigation to quantify stress-induced changes in proDYN mRNA in the SON. In addition, there are limited studies of changes in proDYN mRNA in normotensive rats following exposure to any stressors. Neurons containing dynorphin in the CNS of normotensive rats such as WKY have been found to respond to particular stressors that alter the osmotic properties of the blood, such as administration of a 2% NaCl solution for up to 12 days (Lightman & Young, 1987). In this study, proDYN, CRF, vasopressin and oxytocin mRNA levels were found to be increased in the SON during chronic 2% NaCl administration (Lightman & Young, 1987). In addition, restraint has activated oxytocin-containing neurons within the rat SON (Miyata *et al.*, 1995). However, stressors such as restraint, i.p. hypertonic saline and i.p. isotonic saline did not activate vasopressin mRNA in the SON of normotensive rats (Lightman & Young, 1987; Harbuz *et al.*, 1994; Herman, 1995). Considering that the present study did not report any effect of restraint on proDYN mRNA levels in the SON of normotensive rats, it is clear that the various transmitter and hormonal systems within the SON have unique sensitivities to different stressors, and the increase in mRNA synthesis for one neuropeptide may not necessarily reflect the status of another transmitter system. As such, it appears that a complex stressor-dependent interaction between neuropeptides present in the neurons of the SON occurs during exposure to stress.

Given that a significantly reduced basal expression of proDYN mRNA has already been shown to exist in brain sections of SHR (see section 3.4.3.1), the restraint-induced increase in proDYN mRNA expression in the SON demonstrates that the dynorphin system is still functional in the SON of SHR. Furthermore, it appears that the dynorphin-containing neurons of the SON have a clearly elevated sensitivity to stress in the hypertensive SHR when compared to WKY. The consequences of increased proDYN mRNA levels in the SON during stress may be manifested as changes in the release of hormones such as oxytocin and vasopressin. Kjaer and

co-workers (Kjaer *et al.*, 1995b) demonstrated that the previously reported activation of oxytocin neurons within the SON (Miyata *et al.*, 1995) precedes an elevation in plasma levels of oxytocin during exposure to restraint stress. This study was performed using normotensive rats, and it is presently unknown how the oxytocin system of SHR rats responds to acute and chronic restraint. Furthermore, the disparity in results previously published in studies of basal plasma levels of oxytocin in normotensive and hypertensive male rats clouds the possible relationship between reduced proDYN mRNA levels in the SON of SHR and plasma oxytocin concentrations (Morris *et al.*, 1983; Rosella-Dampman *et al.*, 1985). In order to gain a better understanding of the possible regulatory relationship between the dynorphin-containing neurons of the SON and plasma oxytocin levels in SHR during stress, a number of detailed experiments are required. Using both SHR and WKY, a study comparing proDYN mRNA levels in the SON,  $\kappa$ -opioid receptor density in the SON and neurohypophysis and plasma oxytocin levels during exposure to acute and chronic restraint would provide an indication of the relationship between dynorphin and oxytocin during stress. Immunohistochemistry can also be employed to look at the colocalisation of dynorphin and oxytocin in SON neurons of SHR that are activated by restraint stress. Pharmacological antagonism of  $\kappa$ -opioid receptors using indirect (i.v. or i.c.v.) or direct (intra-SON) administration of non-selective (naloxone) or  $\kappa$ -selective (nor-binaltorphimine) antagonists and quantification of plasma hormones such as oxytocin, as well as measurement of cardiovascular parameters, during exposure to restraint stress would also provide functional evidence of an association between oxytocin and dynorphins in SHR.

Plasma vasopressin concentrations were not changed by restraint in normotensive rats, a result supported by the findings of the present study where no changes in proDYN mRNA were observed in the SON of WKY (Kjaer *et al.*, 1995b). However, an investigation of the restraint-induced plasma levels of vasopressin is warranted in SHR rats who already exhibit an aberrant vasopressin system (Morris *et al.*, 1983; Rosella-Dampman *et al.*, 1985). Thus, a defective vasopressin system in the basal state may not function as expected when SHR rats are exposed to restraint. Experiments similar to those described above for oxytocin would provide an adequate investigation of the modulation of vasopressin release during restraint and other psychological stressors by dynorphin, particularly at the level of the SON.

Another hypothalamic nucleus, the VMH, was sensitive to both acute and chronic restraint in both strains. In WKY, proDYN mRNA increased significantly after 1, 3 and 5 days of the restraint paradigm and was approaching basal levels after 10 sessions of restraint. In contrast, 1 and 10 periods of restraint induced a significantly elevated level of expression of proDYN

mRNA in the VMH of SHR, with non-significant increases observed on days 3 and 5 of the restraint paradigm. As the levels of proDYN mRNA were still elevated after 10 sessions of restraint in the VMH of SHR, the input to these neurons may not be changing with chronic stress, while in WKY, the input to VMH neurons appears to have returned to prestress levels after 5 days of exposure to the restraint paradigm. Previous studies have reported a mild activation of VMH neurons by psychological stressors such as restraint and swim stress (Cullinan *et al.*, 1995; Cullinan *et al.*, 1996), although it is not known whether these neurons contain dynorphin or proDYN mRNA. Furthermore, targets of efferent neurons of the VMH that contain dynorphin have not been described. Tracing studies have demonstrated that the VMH projects to region such as the ARC, SON, POA, BNST, lateral septum, amygdala (Ce, Me), thalamus, PAG, LC, PB and NTS (Krieger *et al.*, 1979; Canteras *et al.*, 1994), all of which contain populations of  $\kappa$ -opioid receptors (see section 1.3.1.3). Thus, it is possible that the VMH sends dynorphin-containing efferents to some, many or all of these areas.

Another region receiving projections originating in the VMH is the median eminence (Krieger *et al.*, 1979). Lesion of the VMH in rats has been shown to reduce dynorphin-ir in the anterior pituitary, suggesting that the VMH projection to the median eminence may contain proDYN mRNA (Spampinato *et al.*, 1988). If further investigations demonstrate that dynorphin is present in this neuronal projection, then elevated levels of proDYN mRNA observed in the VMH during restraint stress may increase the adenohipophyseal concentrations of dynorphin. While the role of dynorphins in the VMH has not been studied extensively, lesions of the VMH have produced interesting effects in addition to those localised in the adenohipophysis. Following VMH lesion, adrenal sympathetic nerve outflow increased and resulted in enhanced catecholamine release from the adrenal gland (Yoshimatsu *et al.*, 1985). In contrast, the activity of sympathetic nerves innervating brown adipose tissue was reduced by VMH lesion, and this was associated with an increase in food intake (hyperphagia) and increased liver, white adipose tissue and body weights (Tejwani & Richard, 1986; Sakaguchi *et al.*, 1988). Whether opioids localised in the VMH, in particular the dynorphins, have the ability to modulate sympathetic outflow will need to be addressed in future studies. The results will also provide more information on the functional consequences of elevated proDYN mRNA levels in the VMH during exposure to acute and chronic restraint stress.

The DG of SHR exhibited a similar response to restraint as that observed in the SON of SHR, with significantly increased levels of the proDYN transcript detected after each period of restraint stress. The significant increases observed on each day of the restraint paradigm, with 3

sessions of restraint producing the largest elevation of +223%, indicate that the input to the dynorphin-containing neurons in the DG of SHR may not be altering its activity during chronic (10 sessions) exposure to restraint. In contrast, the afferent connections of the DG neurons of WKY were apparently able to adapt and modify their activity to repeated restraint, with 1 and 5 days of the restraint paradigm producing a significant increase in proDYN mRNA levels that returned to basal levels of expression on subsequent exposure to restraint. Furthermore, the relative sizes of the augmented expression of proDYN were much larger in SHR than WKY.

These differences between strains relating to the temporal response of neurons of the DG that contain dynorphin to a psychological stressor are in addition to the significantly reduced basal levels of proDYN mRNA, dynorphin-ir and  $\kappa$ -opioid receptors previously observed in the DG of SHR compared to normotensive controls (Li *et al.*, 1989; McConnaughey *et al.*, 1992; Privette *et al.*, 1994; Wang *et al.*, 1994b; Yin *et al.*, 1997). A significantly elevated level of proDYN mRNA in the DG, as observed at various restraint periods in both strains, may result in functional effects in a number of different systems. As described in section 3.4.3.1, direct intra-DG administration of  $\kappa$ -selective agonists produced a hypotensive and bradycardic response in both WKY and SHR (Wang & Ingenito, 1994a). If changes in the cardiovascular system rapidly follow these alterations in proDYN gene expression, then a hypotension and bradycardia should also be observed. Referring to the study of McDougall and colleagues (McDougall *et al.*, 2000), 60 min of restraint produces a sustained hypertension in SHR that does not adapt or change during the 60 min restraint period, and furthermore, the pressor response is maintained over the entire 10 day restraint paradigm. The consistently elevated expression of proDYN mRNA in the DG of SHR may therefore represent an attempt to adapt and reduce the restraint-induced hypertension towards normal resting levels. However, the hypertensive response still persists, suggesting that there is a much more powerful influence on BP during restraint that is overriding the DG. This influence may originate in one of the many central nuclei associated with central cardiovascular regulation, such as the RVLM (Dampney, 1994).

In WKY, initial exposure to restraint produces a rapid pressor response that gradually returns to baseline levels during the 60 min restraint period (McDougall *et al.*, 2000). Therefore, the increase in proDYN mRNA expression in the DG of WKY on the first day of the restraint paradigm may also be an attempt to cope with the pressor response produced by restraint. However, the fact that the temporal pattern of the BP response to restraint remains relatively constant, while there are fluctuations in the expression of proDYN mRNA in the DG during the

10 day restraint paradigm in WKY suggests that another central region is controlling BP and HR during stress, with the DG having a relatively insignificant role.

While the evidence discussed above indicates that the DG may not be integral to cardiovascular modulation during stress, the DG may be involved in the central modulation of another function. In the rat, the dynorphin system in the DG has also been implicated in learning and memory, with  $\kappa$ -opioid receptor agonists such as dynorphin B inhibiting the formation of memory (Sandin *et al.*, 1998). Thus, restraint-induced elevations in proDYN mRNA levels in the DG and the subsequent release of the proDYN peptide products in the hippocampus would presumably inhibit memory formation and possibly attenuate the ability of the rat to cope and adapt to the restraint stimulus. The results of the present study, where proDYN mRNA expression in the DG of SHR does not adapt to chronic restraint, together with the findings of McDougall and colleagues (McDougall *et al.*, 2000) suggest that the potential increase in the release of the dynorphins in the hippocampus may be having detrimental effects on memory and adaptive behaviours during exposure to chronic restraint. Once more, the fluctuations in proDYN mRNA expression in the DG of WKY during chronic restraint make an association between an increased release of dynorphins in the hippocampus and memory formation harder to evaluate. Further studies, where the restraint-induced release of the dynorphins in the hippocampus of SHR and WKY are measured would provide a clearer insight into the role of dynorphin-containing efferent projections from the DG to the hippocampus during stress.

Previous immunohistochemical and ISHH studies have demonstrated that the CPu can be activated by psychological stressors such as restraint (Cullinan *et al.*, 1995; Cullinan *et al.*, 1996). The present investigation therefore supports these studies, as increases in proDYN mRNA expression were detected in the CPu of both normotensive and hypertensive rats. Furthermore, the temporal response to restraint was significantly different between WKY and SHR, with a modest, significant elevation in proDYN mRNA levels observed only after 5 periods of restraint, while increases of up to +210% were detected in the CPu of SHR throughout the restraint paradigm. As described earlier (see section 3.4.3.1), dynorphin-containing neurons of the CPu project to the substantia nigra and dynorphins in the striato-nigral pathway have modulated a variety of functions, including dopamine release and movement (Fallon *et al.*, 1985; Friederich *et al.*, 1987; Reid *et al.*, 1990; Steiner & Gerfen, 1995).

The involvement of the Ce in the central stress response is controversial, with reports of stress-induced activation of Ce neurons matched by studies reporting no changes in neuronal

activity in the Ce during stress (Honkaniemi, 1992; Chen & Herbert, 1995; Stamp & Herbert, 1999). The present study provides support for a role for the Ce in the response to restraint stress, with proDYN mRNA increasing in the Ce of both WKY and SHR during the restraint paradigm. In WKY, an initial increase in proDYN mRNA expression was followed by a return to baseline levels on subsequent periods of restraint, demonstrating that an adaptive mechanism is functional within this nucleus. A recently published report also found that the Ce was able to adapt to repeated restraint, as demonstrated by a reduced expression of Fos-ir in rats exposed to a 14 day restraint paradigm compared to the acutely-stressed group (Stamp & Herbert, 1999). In contrast, a different temporal response to the restraint paradigm was observed in SHR, with a steady rise in proDYN mRNA levels in the Ce of SHR observed with increasing number of restraint sessions. This result can be interpreted in a number of ways. The increase in proDYN mRNA in the Ce is associated with increase in the length of the stressor, and this pattern may indicate that the role of the dynorphin-containing neurons is greater during times of chronic stress. Alternatively, the afferent input to the Ce may not be adapting to repeated restraint, and this dysfunction may be causing the increase in proDYN mRNA levels in the Ce of SHR.

The vast majority of the neurons containing dynorphin localised within the Ce of rats have been shown to possess glucocorticoid receptor-ir (Cintra *et al.*, 1991), suggesting that this region may be integrating emotional and behavioural components of the stress response with the neuroendocrine system. Efferent neurons of the Ce that contain dynorphin-ir project to regions such as the PB, dorsal VTA and substantia nigra (Fallon *et al.*, 1985; Code & Fallon, 1986). The Ce also projects to regions containing  $\kappa$ -opioid receptors such as the PVN, PAG, LC and NTS (Gray *et al.*, 1989; da Costa Gómez & Behbehani, 1995; Pickel *et al.*, 1995; Van Bockstaele *et al.*, 1996), and tracing studies combined with immunohistochemical detection of dynorphin-ir would provide more information regarding the phenotype of these projections.

The studies into the functional effects of the dynorphins in the Ce have been limited to feeding and reward (Berman *et al.*, 1994; Hurd *et al.*, 1999). However, dynorphin has the capacity to modulate additional functions attributed to the Ce that may be more significant during stress. With evidence implicating the Ce in the conditioned fear response, regulation of CRF and ACTH release during stress and production of stress-induced analgesia (Beaulieu *et al.*, 1987; LeDoux *et al.*, 1988; Beaulieu *et al.*, 1989; Fox & Sorenson, 1994), changes in proDYN mRNA expression during stress may have an influence on any one of these integral components of the central stress response.

In WKY, no changes in proDYN mRNA were detected in the NTS at any stage during the restraint paradigm. In contrast, chronic (3, 5 and 10 sessions) restraint induced significant elevations in the expression of the proDYN transcript in the NTS of SHR. The results suggest that the dynorphin-containing neurons in the NTS of SHR may be implicated in the neural response to chronic stress exposure, as acute restraint had no effect on proDYN mRNA levels in this region. Equally, the findings of the present study may also be interpreted in a different light, with the lack of changes in proDYN mRNA expression in the NTS of WKY indicating that these neurons have a high threshold of activation during stress. Specifically, it may only be stressful stimuli that are interpreted to have a magnitude above a particular level that have the capacity to activate these neurons and recruit them for the stress response. Consequently, the present restraint stimulus may not represent a significant threat to WKY, so that the dynorphin-containing neurons of the NTS are not required. This hypothesis can be supported by functional and neuroanatomical data, with the cardiovascular system and NTS activity rapidly adapting to a 60 min restraint session (Chen & Herbert, 1995; Stamp & Herbert, 1999; McDougall *et al.*, 2000). In SHR, the observation that chronic restraint increased the expression of proDYN mRNA may indicate that in this particular strain, restraint is perceived as a substantial stressor and requires a larger response. Whether this discrepancy between WKY and SHR in the temporal stress response of neurons containing dynorphin in the NTS is related to elevated blood pressure status or another phenotypic difference between strains remains to be determined.

The NTS neurons containing dynorphin-ir receive vagal afferent input, and can actively participate in the transmission of information from the viscera to higher brain centres. Dynorphin-containing projections arising in the NTS have been shown to terminate in the hypothalamus, BNST and PB (Riche *et al.*, 1990). These projections demonstrate that alterations in proDYN mRNA expression, and the subsequent modulation of neurotransmission originating from the vagus nerve, has the potential to affect the activity of central nuclei important for different components of the stress response.

As detailed in section 3.4.3.1, the intrinsic release of the proDYN peptide products from dynorphin interneurons in the NTS has the potential to decrease EP and reduce HR and respiratory rate (Hassen *et al.*, 1984; Rabkin, 1993). Therefore, if the elevated expression of proDYN mRNA observed in the NTS of SHR results in the increased release of dynorphins and other peptides within the NTS itself, then hypotension, bradycardia and suppressed respiration could be observed during chronic restraint. However, parallels between the cardiovascular response of SHR rats exposed to chronic restraint published by McDougall and colleagues and

proDYN mRNA levels in the NTS of SHR are not very clear (McDougall *et al.*, 2000). In response to the 10 day restraint paradigm, which was the same as that used in the present study, SHR rats exhibited a hypertensive response that did not adapt, either during the 60 min restraint session or at any stage of the 10 day restraint paradigm (McDougall *et al.*, 2000). The temporal response of the HR of SHR to chronic restraint was slightly different than that of BP. Acute exposure to restraint induced an initial tachycardia that remained maximal for almost 30 min, before falling towards baseline values. As the number of restraint sessions increased, the initial tachycardia was maintained for approximately 10 min, and this temporal pattern was similar to that observed in age-matched WKY rats undergoing the same stress paradigm. The findings of McDougall and co-workers therefore suggest that HR, but not BP, in hypertensive rats has the ability to adapt to chronic restraint stress, and provides further evidence for a dysfunctional sympathetic, but not parasympathetic, nervous system in SHR (McDougall *et al.*, 2000). In light of the results of McDougall and colleagues (McDougall *et al.*, 2000), if proDYN mRNA levels in the NTS of SHR are associated with modulation of the cardiovascular system, then a number of processes may be occurring. Firstly, the elevated expression of proDYN mRNA in the NTS of chronically stressed SHR rats may be a successful attempt by the CNS to attenuate the restraint-induced tachycardia. Alternatively, if the dynorphin system in the NTS is involved in the modulation of sympathetic outflow, and hence BP, then the changes in proDYN mRNA in the NTS of SHR may not be having any effect on restraint-induced hypertension. The level of BP during restraint may be controlled by another region, such as the RVLM, which has the capacity to override any influence from regions such as the NTS. Finally, dynorphin neurons in the NTS may not be modulating cardiovascular parameters during restraint, and changes in proDYN mRNA levels in the NTS of SHR may be a result of alterations in the function of one or more physiological systems that have been induced by restraint. Further studies, involving lesions of the NTS, antagonism of  $\kappa$ -opioid receptors within the NTS or intra-NTS application of proDYN antisera in SHR during exposure to stress, may assist in delineating the role of dynorphin-containing neurons within the NTS in the central stress response.

The findings of the present study demonstrate that proDYN mRNA levels are sensitive to perturbation by acute and chronic restraint in both WKY and SHR rats. Comparing both strains clearly shows that the dynorphin system of hypertensive SHR is a great deal more sensitive to restraint stress, and generally does not adapt to the chronic restraint paradigm. Moreover, in regions where basal proDYN mRNA levels were significantly lower in SHR compared to WKY (SON, DG, CPu, NTS), acute and chronic restraint produced far greater increases in proDYN

mRNA expression of up to +263% (SON) in SHR than in the same regions in the WKY CNS. Therefore, these regions also appear to be hyper-responsive to the restraint stress stimulus, even though deficits in expression of the proDYN transcript exist in the basal state.

### 3.4.4 PREPRO-GAL mRNA EXPRESSION

#### 3.4.4.1 Strain comparison of basal expression

Prepro-GAL mRNA expression was detected throughout the CNS of WKY in sections containing the PVN, SON, Ce, LC and NTS, and this distribution profile of prepro-GAL mRNA is similar to previous studies (Gundlach *et al.*, 1990; Ryan & Gundlach, 1996). The distribution of prepro-GAL mRNA in the SHR CNS has not been fully described, although one study has detected similar levels of prepro-GAL mRNA expression in the LC and NTS of normotensive and hypertensive rats (Kunkler *et al.*, 1994). To determine if differences in prepro-GAL mRNA expression exist in brain nuclei other than the LC and NTS, the density of prepro-GAL mRNA throughout selected regions of the CNS of SHR was measured.

The basal distribution profile of prepro-GAL mRNA was similar in the WKY and SHR strains, but differences in prepro-GAL mRNA density were shown to exist in two brain nuclei. Prepro-GAL mRNA levels were significantly lower in the SON of SHR than WKY, a region known to participate in the regulation of blood volume and pressure and water-electrolyte balance (Meister *et al.*, 1990a; Dun *et al.*, 1995). In the rat SON, GAL has been shown to coexist with vasopressin (Skofitsch *et al.*, 1989), a neuropeptide with a significant role in water-electrolyte balance and cardiovascular regulation (Berecek, 1986). Moreover, central (i.c.v. and intra-cisternal) administration of GAL has been shown to attenuate the release of vasopressin that was stimulated by dehydration and polyethylene glycol-induced hypovolaemia (Kondo *et al.*, 1991; Kondo *et al.*, 1993). However, in the anaesthetised, unstressed rat, Balment and colleagues demonstrated that i.c.v. administration of GAL had no effect on basal vasopressin levels, HR or MAP, suggesting that the actions of GAL on the cardiovascular and vasopressin systems are not utilised during normal resting conditions (Balment & al Barazanji, 1992). These studies were carried out in normotensive rats, and it has been reported that SHR have elevated plasma levels of vasopressin when compared to WKY (Morris *et al.*, 1983; Van Tol *et al.*, 1988). Additional comparisons of vasopressin mRNA content in the SON of WKY and SHR have also

revealed that SHR have a higher expression of vasopressin mRNA (Van Tol *et al.*, 1988). Thus, it is not clear how the modulatory relationship between GAL and vasopressin functions in SHR. Further experiments that involve a detailed investigation of GAL and vasopressin in the SON of SHR and subsequent comparison with normotensive rats are required. Intra-SON administration of GAL could be combined with a variety of functional techniques, such as the quantification of changes in plasma vasopressin in SHR and WKY, as well as electrophysiological measurement of SON neuronal activity in both SHR and WKY.

In agreement with previous studies (Kunkler *et al.*, 1994), there were no significant differences in expression of prepro-GAL mRNA between rat strains observed in the LC and NTS. However, prepro-GAL mRNA levels were significantly increased in the RVLM of SHR when compared to WKY rats. The RVLM is known to have an integral role in the baroreceptor reflex loop via modulation of sympathetic vasomotor outflow (Dampney, 1994; Minson *et al.*, 1996). Alterations in the function of the RVLM can produce changes in baroreceptor reflex sensitivity, and these changes are thought to be implicated in genetic hypertension (Boone & McMillen, 1994b; Kubo *et al.*, 1995). There have been no investigations of the functional role of GAL in the RVLM of the rat, although one study has demonstrated that GAL inhibits the stimulation-evoked release of NAdr in medulla oblongata slices (Tsuda *et al.*, 1992). When comparing SHR with WKY, Tsuda and colleagues (Tsuda *et al.*, 1992) reported an attenuation of the GAL-mediated inhibition of NAdr release in SHR. As discussed in section 1.3.2.3, little is known about the cardiovascular actions of GAL in the medulla. The only functional study reported that GAL receptor stimulation in the rat NTS suppressed the baroreceptor reflex response elicited by phenylephrine (Shih *et al.*, 1996). The same study also demonstrated that intra-NTS injection of a GAL antiserum could suppress the baroreceptor reflex response induced by electrical stimulation of the LC. Furthermore, GAL did not appear to tonically modulate HR or BP at the level of the NTS (Shih *et al.*, 1996), and further studies are required to determine whether administration of GAL, or a GAL receptor antagonist, directly into the RVLM has the capacity to modify resting HR and BP.

GAL-ir has been shown to co-exist with TH-ir in more than 80% of LC neurons (Holets *et al.*, 1988). However, an early study by Melander and co-workers (Melander *et al.*, 1986c) did not report any colocalisation of GAL-ir with TH-ir or phenylethanolamine *N*-methyl transferase (PNMT)-ir in the RVLM. A major group of PNMT-ir cells called the C1 cell group project from the RVLM to vasomotor sympathetic preganglionic neurons in the IML of the spinal cord (Dampney, 1994). This neuronal cell group utilises glutamate as its primary neurotransmitter

and stimulation of these neurons produces a hypertensive response (Bachelard *et al.*, 1990). Although previous studies have reported that GAL inhibits catecholaminergic neurotransmission (Tsuda *et al.*, 1992), it is unlikely that GAL inhibits the activity of these neurons as this would result in a decrease in BP. Another possible site of action for GAL are the non-C1 spinally projecting barosensitive neurons that originate in the RVLM (Lipski *et al.*, 1995). A detailed study that investigates whether GAL is contained in these non-C1 spinally projecting neurons could be achieved using retrograde or anterograde tracing to identify RVLM neurons that project to the spinal cord, with subsequent immunohistochemical processing of sections containing the RVLM for GAL-ir and TH-ir using fluorescent markers to determine the phenotype of these neurons.

Increased cholinergic activity and acetylcholine (ACh) release in the RVLM of SHR has been implicated in the maintenance of hypertension in these rats (Kubo *et al.*, 1995), as injection of cholinergic agonists into the RVLM results in increased BP (Kubo *et al.*, 1995). The effect of GAL on ACh neurotransmission within the RVLM is unknown, but previous evidence demonstrates that GAL inhibits cholinergic transmission in the hippocampus (Fisone *et al.*, 1987; Palazzi *et al.*, 1988). If GAL inhibited cholinergic transmission in the RVLM, then a decrease in BP would be produced. While indirect evidence indicates that a direct inhibitory action of GAL on cholinergic transmission or bulbospinal neurons in the RVLM would be unlikely, elevated levels of GAL in the RVLM could increase BP through disinhibition of an inhibitory interneuron. Inhibition of  $\gamma$ -aminobutyric acid (GABA) neurotransmission in the RVLM resulted in a pressor response (Willette *et al.*, 1984a; Kubo & Kihara, 1987), but the presence of GAL receptors on these neurons or the relationship between GAL and GABA in the RVLM has not been investigated. Thus, while there are a number of possible mechanisms through which GAL could modulate the activity of RVLM neurons, it is not until the completion of studies where cardiovascular and neurochemical changes are measured following intra-RVLM GAL microinjection that clearer insights into the role of GAL in the RVLM will be gained.

#### 3.4.4.2 Effects of restraint stress

Exposure to restraint stress is known to have widespread effects throughout the CNS, as shown by changes in expression of *c-fos* mRNA and/or Fos protein (Melia *et al.*, 1994; Chen & Herbert, 1995). However, the role of GAL in the neural response to acute and chronic restraint

stress has not been reported. As such, this study is the first investigation of changes in prepro-GAL mRNA in the rat CNS following exposure to restraint stress. A significant increase in prepro-GAL mRNA expression was detected in the WKY Ce after one period of restraint. This elevated expression of prepro-GAL mRNA in the Ce had returned to basal levels by the third day of the restraint paradigm, suggesting the presence of habituation in the WKY.

Exposure of WKY rats to repeated periods of restraint stress did not induce any significant changes in prepro-GAL mRNA in other brain nuclei, although an increase of approximately 140% was recorded in the NTS after 5 and 10 sessions of restraint. This small increase in prepro-GAL mRNA expression in the NTS indicates that GAL may be involved in the response to chronic stress at the level of the NTS. The NTS is the termination site of primary vagal afferents from the viscera, including cardiovascular, respiratory and gustatory systems (Lawrence & Jarrott, 1996). Although stress causes a widespread activation of physiological systems, few studies have detected significant changes in the rat NTS following restraint stress (Krukoff & Khalili, 1997; Stamp & Herbert, 1999).

In WKY, the temporal nature of changes in prepro-GAL mRNA expression in the Ce, SON and other brain nuclei over the 10 day restraint paradigm suggests that the normotensive WKY rat has the ability to adapt to chronic restraint. This adaptation to restraint is particularly apparent in the Ce of WKY, where the initial increase in prepro-GAL mRNA expression after one restraint session returns to basal levels following subsequent exposure to restraint. In contrast, the expression of prepro-GAL mRNA in the Ce and SON of SHR rats shows progressive increases as the number of restraint sessions increase. More importantly, a comparison of the temporal response between strains revealed that there was a significant interaction between the strain and exposure to restraint in the Ce and SON. This was clearly apparent when graphically comparing the expression of prepro-GAL mRNA in these nuclei for each strain for each period of restraint stress (Figure 3.16). In addition, there was a progressive decrease in prepro-GAL mRNA expression in the RVLM of SHR with increasing exposure to restraint; however, this trend was not significant. These patterns of prepro-GAL mRNA expression in the SON and Ce do not show any indication of returning towards baseline levels, suggesting that the SHR has an impaired ability to adapt to repeated restraint stress. These results are supported by a number of previous studies that have also reported an impaired ability of SHR rats to cope with different stressors (Armario *et al.*, 1995; Gómez *et al.*, 1996; Li *et al.*, 1997). However, this inability to adapt to restraint stress in SHR appears to be specific to the Ce

and SON, as changes were not observed in any other brain nuclei where prepro-GAL mRNA expression was quantified.

The largest changes in prepro-GAL mRNA expression were seen in the Ce and SON of SHR rats, with elevations of up to 300% after 10 sessions when compared to day 0 levels. The SON appears to play an important role in the response to restraint stress. For example, neurons within the SON of rats have been activated by exposure to restraint or immobilisation (Imaki *et al.*, 1993; Miyata *et al.*, 1995). However, there is a paucity of information regarding changes in specific neurotransmitters in the SON induced by restraint stress. An increase in CRF and nitric oxide synthase (NOS) mRNA expression in the SON following periods of restraint has been reported (Luo *et al.*, 1994; Krukoff & Khalili, 1997), with restraint inducing a larger increase in NOS mRNA expression when compared to an environmental stressor. No habituation of the elevated expression of prepro-GAL mRNA was observed in the SON in the present study following periods of chronic restraint; instead, expression increased as the number of restraint sessions increased.

The activation of the SON by stress depends on the nature of the stressor. Stressors such as haemorrhage activate the SON (Feuerstein *et al.*, 1985), whereas cold and isolation stress do not increase vasopressin mRNA in the SON (Angulo *et al.*, 1991). In addition, the SON appears to play a much more important role in response to stressors that alter the osmotic properties of the blood. Stressors such as i.p. administration of hypertonic saline and chronic administration of 2% NaCl induce changes in *c-fos* expression and increase mRNA expression of many neurotransmitters within the SON (Lightman & Young, 1987; Young & Lightman, 1992; Luo *et al.*, 1994).

The Ce has been reported as an integral part of the centrally-mediated stress response to a number of stressors, including restraint (Beaulieu *et al.*, 1987; Roozendaal *et al.*, 1997). This is reinforced by the results of this study, as prepro-GAL mRNA expression in the Ce was activated by acute restraint in WKY and by chronic restraint in SHR. Furthermore, while the neurons containing prepro-GAL mRNA have the ability to adapt to repeated restraint in WKY, similar neurons in the Ce of SHR appear to lack this ability to cope with chronic restraint. Even though this study demonstrates that restraint can perturb neurons within the Ce containing prepro-GAL mRNA, there have been studies that report no induction of *c-fos* mRNA expression (Chen & Herbert, 1995) or CRF mRNA expression in the Ce following acute or chronic restraint (Pacak *et al.*, 1996). There are a number of possible explanations for this discrepancy in results. The

contrasting results may indicate that slight changes in stressor conditions can affect the response to stress, as other stressors have induced neurochemical changes within the Ce (Beaulieu *et al.*, 1987; Tkacs *et al.*, 1997). In addition, expression of *c-fos* mRNA within a nucleus gives a general indication that the nucleus has been activated by the stimulus (restraint). The lack of *c-fos* mRNA expression reported by Chen and colleagues may indeed represent a decrease in activity of Ce neurons, which may equally lead to the changes in prepro-GAL mRNA expression observed in this study (Chen & Herbert, 1995).

Acute restraint stress induced a significant decrease in prepro-GAL mRNA expression in the LC of SHR that returned to control levels as the number of restraint sessions increased. GAL is colocalised with TH in 80% of neurons within the LC (Holets *et al.*, 1988), and stress has been shown to activate the NAdr neurons in the LC (Lachuer *et al.*, 1994). Furthermore, it appears that the changes in prepro-GAL mRNA expression in the LC following exposure to stress are stressor-specific, as not all stressors increase prepro-GAL mRNA levels (Austin *et al.*, 1990; Holmes *et al.*, 1995). In addition, the results of this study suggest that the GAL system within the LC may adapt to repeated restraint. Although some studies have supported the hypothesis of habituation to stress within the LC (Lachuer *et al.*, 1994; Watanabe *et al.*, 1994; Conti & Foote, 1996), others have not (Chen & Herbert, 1995), demonstrating the variable influence of different stressors on the stress response in this pontine nucleus.

### 3.4.5 PREPRO-NPY mRNA EXPRESSION

#### 3.4.5.1 Strain comparison of basal expression

Expression of the mRNA encoding the NPY precursor, prepro-NPY, was detected in many discrete nuclei throughout the CNS of normotensive (WKY) and hypertensive (SHR) rat strains. The distribution of prepro-NPY mRNA was similar between strains, with detectable levels of the transcript localised in regions such as the cortex, ARC, amygdala, hippocampus, Ret, LC, NTS, sp5 and VLM. Previous reports have described a similar distribution of prepro-NPY mRNA throughout various levels of the normotensive rat CNS (Gehlert & Wamsley, 1987; Morris, 1989; Houdeau & Boyer, 1994; McLean *et al.*, 1996). In agreement with the above studies, a high level of prepro-NPY mRNA expression was observed in the hypothalamic ARC in both strains. A comparison between WKY and SHR demonstrated that hypertensive SHR contained a

significantly greater density of prepro-NPY mRNA in the ARC compared to WKY, a result also reported by previous studies (McLean *et al.*, 1996; Krukoff *et al.*, 1999). In addition, elevated NPY levels were detected in the ARC of SHR compared with two normotensive rat strains, the WKY and Long-Evans (Clark *et al.*, 1991). In contrast, Higuchi and colleagues (Higuchi *et al.*, 1993) reported similar levels of prepro-NPY mRNA in the hypothalamus of WKY and SHR. Northern blot analysis was used to measure prepro-NPY mRNA content of the hypothalamus in the Higuchi study (Higuchi *et al.*, 1993), and as such, they were unable to delineate the prepro-NPY mRNA content of the different hypothalamic subnuclei such as the ARC.

The ARC is known to contain a high concentration of glucocorticoid receptors, with glucocorticoid receptor-ir present in the majority, if not all of the NPY-ir neurons in the ARC (Ceccatelli *et al.*, 1989a; Cintra *et al.*, 1991). Increased levels of circulating glucocorticoids have been shown to increase levels of prepro-NPY mRNA in the ARC (Larsen *et al.*, 1994b). The concentration of circulating glucocorticoids, such as corticosterone, have been shown by many studies to be increased in the young SHR compared to normotensive rats of a similar age (Hattori *et al.*, 1986; Hashimoto *et al.*, 1989a). However, there have been conflicting reports regarding the basal levels of circulating corticosterone in adult SHR compared to normotensive controls, with studies detecting increased (Iams *et al.*, 1979; Sowers *et al.*, 1981) or decreased (DeVito *et al.*, 1981; Wexler & McMurtry, 1982; Imaki *et al.*, 1998) levels of plasma corticosterone. As the expression of prepro-NPY mRNA has not been measured in the ARC of young SHR, it is unknown whether elevated plasma corticosterone observed in developing SHR has any impact on prepro-NPY mRNA levels in the ARC. Furthermore, future studies also need to measure prepro-NPY mRNA levels in the ARC of adult SHR and correlate these with plasma corticosterone levels in the SHR at the time of the experiment.

At present, there have been no studies that have attempted to determine the neurochemical consequences of increased prepro-NPY mRNA expression in the ARC of SHR. The potential implications of increased NPY peptide synthesis in the ARC of SHR are widespread, as the ARC has been reported to send efferent projections to many nuclei involved in autonomic regulation, such as the PVN, LC and NTS (Bai *et al.*, 1985; Chronwall, 1985; Sim & Joseph, 1991). Although the projection from the ARC to the PVN has been reported to contain NPY (Bai *et al.*, 1985), it appears that increased prepro-NPY mRNA in the ARC may not translate into increased levels of NPY-ir in the PVN, as similar levels of NPY-ir have been reported in the PVN of WKY and SHR (Maccarrone *et al.*, 1986; Clark *et al.*, 1991). However, in the studies by Maccarrone (Maccarrone *et al.*, 1986) and Clark (Clark *et al.*, 1991), the PVN was microdissected from

WKY and SHR brains and the results were expressed as the NPY-ir content of the entire PVN. Consequently, the concentration of NPY-ir in specific subregions of the PVN could not be defined. Future experiments where NPY-ir is quantified and compared on consecutive slide-mounted sections of the PVN would assist in delineating potential differences between WKY and SHR in the parvocellular and magnocellular subnuclei of the PVN.

The ARC may be able to directly influence BP via the NTS, as NPY has induced decreases in both BP and HR following microinjection into the NTS (Takesako *et al.*, 1994; Morris *et al.*, 1997; Yang *et al.*, 1997). Comparisons between strains of the cardiovascular effects induced by intra-NTS microinjection of NPY have produced contrasting results. Takesako and colleagues reported a significantly reduced hypotensive response following injections of NPY into the NTS of SHR compared to WKY (Takesako *et al.*, 1994). In contrast, Morris and co-workers demonstrated that the magnitude of the hypotensive and bradycardic effects following intra-NTS administration of NPY were similar in SHR and WKY (Morris *et al.*, 1997). In another study, lower doses (0.2 – 1 pmol) of NPY were injected directly into the NTS (Yang *et al.*, 1997). Yang and colleagues reported an increased potency of NPY to elicit a hypotensive response in SHR compared to WKY (Yang *et al.*, 1997), a finding that was different to the results of both Takesako (Takesako *et al.*, 1994) and Morris (Morris *et al.*, 1997). Although the results of these three studies are contradictory, they demonstrate that differences in the NPY system exist between SHR and WKY at the level of the NTS, and these may be related to elevated prepro-NPY mRNA levels in the ARC of SHR.

In the present study, comparison between SHR and WKY revealed that the expression of prepro-NPY mRNA was similar in all other central nuclei, supporting the results of previous studies (McLean *et al.*, 1996; Krukoff *et al.*, 1999). However, Higuchi and colleagues reported a significant decrease in prepro-NPY mRNA in the cerebral cortex of adult SHR compared to WKY (Higuchi *et al.*, 1993). There are a number of protocol differences between the present thesis and the study conducted by Higuchi and co-workers that may account for the discrepant results (Higuchi *et al.*, 1993). As described earlier, Higuchi and colleagues (Higuchi *et al.*, 1993) used a northern blot analysis of the grossly dissected cerebral cortex. Therefore, the significantly reduced expression of prepro-NPY mRNA observed in SHR in Higuchi (Higuchi *et al.*, 1993) could be localised to any number of cortical regions where prepro-NPY mRNA has been detected, such as the different layers of the cerebral cortex, the Ent and Pir. In the present study, the resolution of the autoradiographic images did not enable delineation of the different cortical regions. In addition, as a limited region of the cortex at the level of the hypothalamus

and amygdala was sectioned and processed for prepro-NPY mRNA in the present study, differences in expression of prepro-NPY mRNA between strains may exist in cortical regions at other anatomical levels of the CNS, such as the Ent, medial prefrontal and occipital cortex.

#### 3.4.5.2 *Effects of restraint stress*

Evidence exists to support a role for NPY in the central response to stress, including restraint (see section 1.3.3.3). A number of studies have examined alterations in prepro-NPY mRNA expression in selected central nuclei following exposure to restraint stress. Krukoff and colleagues utilised ISHH with slide-mounted brain sections to study the ARC, NTS and VLM (Krukoff *et al.*, 1999), while studies conducted by Thorsell and colleagues used homogenates or extracts from the amygdala, striatum, hypothalamus and cortex (Thorsell *et al.*, 1998; Thorsell *et al.*, 1999). In the latter studies by Thorsell and colleagues, the regions that were analysed consist of many subnuclei that have diverse roles in the regulation of many functions. Thus, any changes that may be detected will be global changes for the entire region, and subtle, specific alterations in prepro-NPY mRNA expression induced by restraint are likely to be overlooked in subnuclei of the amygdala, cortex and hypothalamus. The present study has therefore sought to quantify the expression of prepro-NPY mRNA in specific nuclei in the forebrain, pons and medulla following acute and chronic restraint stress using ISHH. Furthermore, the current study also compared prepro-NPY mRNA levels in the CNS of normotensive and hypertensive rats after exposure to acute and chronic restraint.

In the ARC of WKY, acute restraint caused a significant increase in prepro-NPY mRNA expression that had returned to basal levels of expression by the third day of the restraint paradigm. After 5 and 10 periods of restraint, there was no significant change from normal levels of prepro-NPY mRNA expression in the ARC of WKY. This response pattern demonstrates that in WKY, ARC neurons containing prepro-NPY mRNA have altered their activity as the number of restraint sessions increased. In the ARC of SHR, 3 sessions of restraint produced a significant increase in prepro-NPY mRNA expression that was of a similar magnitude to the increase in prepro-NPY mRNA observed in the WKY ARC after 1 session of restraint. Previously published studies have reported significant increases in prepro-NPY mRNA levels in the ARC of normotensive rats exposed to 2 hours of immobilisation for 1, 6 or 14 days (Makino *et al.*, 1999; Makino *et al.*, 2000). Immobilisation represents a greater psychological

stress when compared to restraint, and this may be reflected in the sustained increases in prepro-NPY mRNA expression in the ARC during 14 days of consecutive exposure to immobilisation. It is not known whether increased synthesis of prepro-NPY mRNA in the ARC represents an increase in production of the NPY peptide, as NPY-ir decreased in the ARC of rats exposed to restraint (Pralong *et al.*, 1993).

In the present study, 1 hour of restraint resulted in increased expression of prepro-NPY mRNA in the ARC after 1 (WKY) or 3 days (SHR) of exposure. This result is in contrast to an earlier report, where prepro-NPY mRNA levels decreased significantly in the ARC of normotensive (WKY and SD) and hypertensive (SHR) rats subjected to 1 day of restraint (Krukoff *et al.*, 1999). The restraint paradigm used in the Krukoff study in 1999 (Krukoff *et al.*, 1999) consisted of 1 hour in a restraint tube, followed by 1 hour of rest, then 1 hour of restraint and 1 hour of rest. In total, this equated to 2 hours of restraint during the 4 hour paradigm before the rat was killed. In comparison to the 1 hour of restraint used in the present study, there is a clear difference between studies in the time allowed for expression of prepro-NPY mRNA in response to the restraint stimulus. While it is not known how rapidly changes in prepro-NPY mRNA expression occur after the onset of the restraint stimulus, significant increases and decreases in prepro-ENK mRNA expression have been detected after 2 min of restraint (Boone & McMillen, 1994a). After 30 min of restraint, these initial responses in prepro-ENK mRNA expression increased, decreased or remained the same depending on the nucleus (Boone & McMillen, 1994a). While data exist for rapid changes in synthesis of precursor mRNA following restraint, previous reports have also described changes in the physiological response to restraint over short time periods. McDougall and colleagues showed that after WKY rats were placed in a perspex restraint tube, the initial increase in BP and HR lasted for approximately 30 min before stabilising at a level slightly above baseline values (McDougall *et al.*, 2000). These studies of Boone (Boone & McMillen, 1994a) and McDougall (McDougall *et al.*, 2000) suggest that there is enough time for modification and adaptation of the initial response exhibited by neurons expressing prepro-NPY mRNA during the 4 hour restraint paradigm used by Krukoff and co-workers (Krukoff *et al.*, 1999), and even within the 1 hour restraint period in the present study. It is therefore possible that the significant increase in prepro-NPY mRNA expression observed in the ARC after 1 hour of restraint in this study precedes a significant decrease in expression of the prepro-NPY transcript following a 4 hour restraint paradigm. Thus, plasticity in the form of changes in expression of prepro-NPY mRNA can be observed during consecutive hours of restraint on the same day and following exposure to 1 hour of restraint over consecutive

days. As such, the true time course of expression of prepro-NPY mRNA during restraint requires a more detailed investigation with measurements of prepro-NPY mRNA levels ranging from a few min to many hours after the onset of restraint over a number of days.

A slower response to restraint occurs in the ARC of SHR compared to WKY, with differences in the number of sessions of restraint required to elicit a significant increase in prepro-NPY mRNA expression. The underlying cause of this altered response is unknown, but may be related to the increased basal expression of prepro-NPY mRNA in the ARC of SHR compared to WKY. In addition, the response of the HPA axis, in particular the plasma concentrations of corticosterone following restraint may have an impact on prepro-NPY mRNA levels in the ARC. In the present study, plasma levels of corticosterone and ACTH were not quantified. Literature reports of the effect of stress on the HPA axis of SHR compared to normotensive rats have been contradictory. Studies have shown that the various components of the HPA axis of SHR were more sensitive to stressors including restraint and immobilisation compared to normotensive controls (McMurtry & Wexler, 1981; Imaki *et al.*, 1998). In contrast, immobilisation elicited greater increases in plasma corticosterone in WKY compared to SHR (Sowers *et al.*, 1981). Thus, to provide an accurate assessment of the relationship between hypertension and activation of the HPA axis and the central NPY system following restraint, indices of NPY neurochemistry and the HPA axis need to be measured in the same group of hypertensive rats that have been exposed to restraint.

The ARC appears to negatively modulate the HPA axis, as lesion of the ARC resulted in an elevated release of ACTH and corticosterone following exposure to stress (Magarinos *et al.*, 1988; Larsen *et al.*, 1994c). The involvement of NPY in the negative relationship of the ARC with the HPA axis is not clear. The ARC is known to project to the anterior pituitary (Chronwall, 1985), but Vanhatalo and colleagues (Vanhatalo & Soinila, 1996) demonstrated that these projections did not contain NPY. In support of the lack of NPY-containing fibres in the anterior pituitary, Small and co-workers (Small *et al.*, 1998) reported that NPY had no effect on the basal or CRF-stimulated release of ACTH from isolated anterior pituitary corticotrophs. An alternative site where the ARC could modulate the HPA axis is the PVN, a region where afferent NPY projections from the ARC have been shown to terminate in close proximity to CRF-ir neurons (Liposits *et al.*, 1988). Functional results suggest that the CRF-ir neurons in the PVN may be modulated by NPY, as intra-PVN administration of NPY results in increased plasma levels of ACTH and corticosterone in unstressed rats (Wahlestedt *et al.*, 1987). Although a similar study has not been repeated in stressed rats, the results in unstressed rats (Wahlestedt *et*

*al.*, 1987) suggest that at the level of the PVN, NPY may not mediate the negative modulatory effect of the ARC on the HPA axis. However, as the ARC contains many neuromodulators other than NPY (Chronwall, 1985), it is likely that one of these peptides may have a principal role in the inhibition of stress-induced HPA axis activation at the level of the PVN.

The role of NPY within the PVN is not limited to modulation of activity of the HPA axis, as NPY has also been implicated in the control of feeding. NPY-ir was increased in the PVN following 3 hours of restraint (Rybkin *et al.*, 1997), while other studies did not detect any alterations in NPY-ir after a restraint period of 30 min (Rivet *et al.*, 1989) or after 60 min immobilisation for 12 consecutive days (Corder *et al.*, 1992). These results suggest that NPY synthesis may increase during exposure to stress for an extended period of time. Restraint that lasts for 3 hours may represent a mild food deprivation stress. There is support for this hypothesis, as studies have demonstrated that food deprivation for 2 or 10 days results in significantly increased levels of NPY within the PVN (Yoshihara *et al.*, 1996). In the present study, 1 hour of restraint is unlikely to result in additional stress caused by food deprivation, and as such the increase in NPY synthesis in the ARC would not be related to this alternative stress.

Prepro-NPY mRNA expression increased significantly in the hippocampus after 3 sessions and in the DG of SHR following 1, 3 and 10 periods of restraint. This study represents the first report of changes in NPY neurochemistry in the hippocampus and DG of SHR following not only restraint, but any form of stress. Previous studies have reported that neurons within the hippocampus and DG of normotensive rats are sensitive to many different stressors, including restraint (Melia *et al.*, 1994; Cullinan *et al.*, 1995; Ryabinin *et al.*, 1995). Restraint did not produce any significant alterations in prepro-NPY mRNA expression in the hippocampus and DG of WKY, suggesting that in normotensive rats, restraint-induced hippocampal activation does not have a major effect on the synthesis of NPY. In contrast, the altered phenotype of SHR may increase the sensitivity of hippocampal NPY-containing neurons to restraint stress. A previous study has also reported an elevated response of NPY-containing neurons to running and sensory stimulation in the hippocampus of SHR (Bucinskaite *et al.*, 1996). A possible factor contributing to this increased sensitivity of the hippocampal NPY system may include an underlying neurochemical difference in NPY receptors within the hippocampus (Chang *et al.*, 1986), in addition to the elevated sensitivity of the sympathetic response to stress observed in this strain (Li *et al.*, 1997). In rats, the hippocampus has been implicated in learning and memory, as well as behavioural state and cardiovascular control (Thomas & Ahlers, 1991; Wang & Ingenito, 1994a; Bitran *et al.*, 1998), suggesting that these functions may be influenced by

differences in the response of NPY-containing neurons to restraint at the level of the hippocampus in SHR.

As described in section 3.3.5.1, the cortex data are representative of all cortical subregions at the level of the hypothalamus and amygdala, including the Cing, RS, Par, Fr and Pir. This was due to the difficulty in clearly identifying the borders of each of the cortical regions using the prepro-NPY hybridisation signal on the forebrain slice. Subjective analysis indicated that there were no discernible differences in levels of prepro-NPY mRNA between each cortical subregion, so the cortex was sampled as a single unit. In the cortex, a similar response to the 10 day restraint paradigm was observed in both strains. Specifically, 5 sessions of restraint induced a significant decrease in prepro-NPY mRNA expression in the cortex of both strains, with no changes evident after 1, 3 or 10 days of the restraint paradigm. Restraint for 1 hour also induced a decrease in prepro-NPY mRNA in the cortex as measured using a solution hybridisation-RNase protection assay (Thorsell *et al.*, 1998). Following analysis of the time course of prepro-NPY mRNA expression, Thorsell and colleagues found that prepro-NPY mRNA was significantly decreased in the cortex 2 and 4 hours after restraint, but similar to control rats at 1 hour and 10 hours post-restraint (Thorsell *et al.*, 1998). These results are in agreement with the present study, where prepro-NPY mRNA was measured immediately after restraint and no significant changes were observed in the cortex after 1 period of restraint in either strain.

Many cortical subregions have been activated by stressors such as restraint, as demonstrated by increased Fos production or increased expression of *c-fos* mRNA in cortical regions such as the medial prefrontal cortex, Cing, Par and Fr (Ryabinin *et al.*, 1995; Yokoyama & Sasaki, 1999). Limited data have been published regarding the role of NPY in many of these cortical regions. However, the extensive neuronal network involving cortical regions such as the RS and Cing suggests that changes in prepro-NPY mRNA following restraint stress may influence neurotransmission in many central nuclei (Takagishi & Chiba, 1991). The medial prefrontal cortex, BL and RVLM have been shown to provide afferent input to regions of the cortex such as the Cing, Pir and RS (Zagon *et al.*, 1994; Dziewiatkowski *et al.*, 1998). Moreover, cortical areas such as the RS and Par project to the thalamus, colliculus and hippocampus, as well as to the medial prefrontal cortex, which contains the medial region of the frontal cortex, anterior Cing and infralimbic cortical subregions (Diorio *et al.*, 1993; Shibata, 1994; Shibata, 1998; Garcia Del Cano *et al.*, 2000).

The medial prefrontal cortex has been associated with modulation of the neuroendocrine and HPA axis component of the stress response, possibly at the level of the PVN (Diorio *et al.*, 1993). The medial prefrontal cortex receives a dense noradrenergic innervation from the LC, a pontine region that is involved in stressor perception, arousal, cardiovascular modulation and neuroendocrine control (Korf *et al.*, 1973; Sved & Felsten, 1987; Butler *et al.*, 1990; Sakaguchi & Nakamura, 1990; Yao *et al.*, 1999; Ziegler *et al.*, 1999; Chowdhury *et al.*, 2000). Previous reports have demonstrated that exposure to restraint results in increased TH mRNA (Smith *et al.*, 1991; Sands *et al.*, 2000) expression in the LC, as well as increased Fos-ir and *c-fos* mRNA expression (Watanabe *et al.*, 1994; Chen & Herbert, 1995; Stamp & Herbert, 1999). The results of the present study demonstrate that prepro-NPY mRNA synthesis was also increased in the LC following restraint, but this response was only observed in SHR and not WKY. The neurons in the LC of SHR containing prepro-NPY mRNA were activated by chronic restraint, with 5 periods inducing a significant increase of +92% and 10 periods of restraint resulting in a non-significant increase. No significant increase or decrease in prepro-NPY mRNA expression was observed in the LC of SHR after 1 or 3 sessions of restraint.

Makino and colleagues (Makino *et al.*, 2000) found that neither acute nor chronic exposure to immobilisation elicited any changes in prepro-NPY mRNA expression in the LC of normotensive rats, supporting the results of the present study. There have been no studies investigating the levels of prepro-NPY mRNA in the LC of SHR after exposure to any stressful stimulus. Furthermore, there have been limited studies of the termination fields of LC neurons containing NPY. One study has demonstrated that small proportions of NPY-ir neurons project from the LC to the cortex, spinal cord and hypothalamus (Holets *et al.*, 1988). Moreover, there have been no detailed studies of the medullary regions receiving NPY-containing efferents from the LC. Thus, it is unclear how alterations in prepro-NPY mRNA in the LC could influence the response to restraint in SHR, as the LC can modulate many physiological functions, including the behavioural, neuroendocrine and cardiovascular state (Sved & Felsten, 1987; Kawasaki *et al.*, 1991; Weiss *et al.*, 1994; Yao *et al.*, 1999; Ziegler *et al.*, 1999). Furthermore, the status of neuroendocrine systems, in particular the plasma levels of corticosterone, appear to have a direct impact on NPY-containing neurons of the LC. Glucocorticoid-ir has been shown to be present in all NPY-ir cells of the LC (Härfstrand *et al.*, 1989), and adrenalectomy significantly reduced the levels of prepro-NPY mRNA in the LC (Watanabe *et al.*, 1995). Thus, further experiments are required to elucidate the cause, and subsequent neural effects, of changes in prepro-NPY mRNA synthesis following chronic restraint in the LC of SHR.

The VLM is representative of both the RVLM and CVLM, as the present study was not able clearly discern the transition between these regions. Despite the grouping of data over a large rostral-caudal range, a significant decrease in prepro-NPY mRNA expression was detected in the SHR VLM after 1 session of restraint. Subsequent exposure to restraint also resulted in a decrease in the VLM of SHR, but these reductions were not significant. In contrast, prepro-NPY mRNA synthesis was not altered by restraint in the VLM of WKY, a result complimented by (Rivet *et al.*, 1989) who also reported no changes in NPY-ir in the VLM of normotensive rats exposed to 30 min of restraint. NPY has been detected in the projection from the A1 cell group of the CVLM to the vasopressin cells of the PVN and SON (Day, 1989). The C1 cell group of the RVLM has been reported to send NPY-containing efferent projections to the hypothalamus, Ce and spinal cord (Tseng *et al.*, 1993; Zardetto-Smith & Gray, 1995; Stornetta *et al.*, 1999), demonstrating that NPY-containing neurons convey information relating to the status of the cardiovascular and sympathetic nervous systems to the hypothalamus, amygdala and spinal cord. A hypotensive challenge resulted in an activation of NPY-ir neurons in the CVLM and RVLM (McLean *et al.*, 1999), suggesting that the decrease in prepro-NPY mRNA expression in the VLM of SHR observed in the present study may reflect a sustained change in BP induced by restraint. In agreement, McDougall and colleagues (McDougall *et al.*, 2000) demonstrated that the SHR strain have a much greater hypertensive response to restraint compared to WKY. In this study, 1 session of restraint induced an increase in BP in SHR that remained elevated for the entire 1 hour restraint period, and this response did not change or adapt following exposure to the same stress on subsequent days. In contrast in WKY, exposure to restraint resulted in an initial rapid rise in BP that was followed by a decrease and eventual plateau approximately 20 mmHg above normal BP levels after 30 min. This hypertensive response to restraint observed in WKY did not alter on subsequent days, suggesting that the WKY rats habituated near-maximally after 1 period of restraint that was maintained for the entire restraint paradigm. Therefore, the significantly altered hypertensive response in SHR compared to WKY (McDougall *et al.*, 2000), coupled with a potential increase in sympathetic activation, may be correlated with the restraint-induced reduction in levels of prepro-NPY mRNA in the VLM of SHR observed in this study.

A period of restraint that lasts for longer than 1 hour may produce different results. Krukoff and colleagues (Krukoff *et al.*, 1999) used a 4 hour restraint paradigm and found that this particular paradigm did not elicit any significant changes in levels of prepro-NPY mRNA in the VLM of normotensive or hypertensive rats. Furthermore, no significant differences were detected between strains, suggesting that the level of activation of NPY-containing neurons in

the VLM were similar in both strains (Krukoff *et al.*, 1999). Combining the results of the study by Krukoff and colleagues (Krukoff *et al.*, 1999) with the current study suggests that the initial decrease in prepro-NPY mRNA synthesis after 1 hour of restraint may return to basal levels of expression after 4 hours. The BP response over this 4 hour restraint paradigm was not measured in any strain, and as such, the levels of prepro-NPY mRNA in the VLM can not be correlated with BP status.

Another two medullary regions, the IOC and sp5, exhibited a significantly different temporal response to restraint in SHR compared to WKY. While prepro-NPY mRNA expression was not altered in these regions of WKY following restraint, prepro-NPY mRNA levels in the IOC and sp5 of SHR increased by +50% after 5 and 10 periods of restraint. Previous studies have reported that neurons within these medullary regions are sensitive to restraint (Cullinan *et al.*, 1995). As there are limited data regarding the role of NPY in these regions, it is unclear what effect differential changes in prepro-NPY mRNA in SHR compared to WKY may have on the neural response to restraint. Neurons containing NPY within sp5 have been shown to project to the peribrachial region (Fodor & Palkovits, 1991) that has previously been implicated in cortical activation and arousal (Datta, 1995; Silvestri & Kapp, 1998). The thalamic NPY-containing projection from the sp5 (Lechner *et al.*, 1993) may have an influence on the central response to whisker stimulation (Williams *et al.*, 1994). Furthermore, known projections to the PAG and PB (Swenson *et al.*, 1984; Cechetto *et al.*, 1985) suggest that sp5 participates in the central integration of peripheral sensory information such as the reflex cardiovascular responses elicited by nociception (Allen *et al.*, 1996).

There have been no detailed investigations of the role of NPY in the IOC. The IOC receives projections from the LC (Fritschy & Grzanna, 1990), cortical regions involved in motor control (Swenson *et al.*, 1989) and sensory trigeminal nuclei (Huerta *et al.*, 1983). Further studies demonstrate that the IOC is part of the neural circuitry controlling movement, with IOC lesions resulting in deficits in motor co-ordination and equilibrium (Rondi-Reig *et al.*, 1997), as well as learning (Dahhaoui *et al.*, 1992). These central connections suggest that the IOC may integrate the behavioural and emotional state of the rat with the physical/motor response to stress and sensory stimulation.

The results of the present study clearly show that many more central regions containing prepro-NPY mRNA were sensitive to perturbation by restraint in the hypertensive SHR compared to WKY. Statistical analysis confirmed that the temporal response to the restraint

paradigm exhibited by a number of regions were significantly different in SHR compared to the age-matched WKY rats. In the forebrain, these regions included the ARC, DG, hippocampus, Ret and cortex. As described in section 3.3.5.2, many of these forebrain nuclei in the SHR exhibited a biphasic response to acute and chronic restraint stress. Specifically, increases in prepro-NPY mRNA expression were detected after 1 and 3 sessions of restraint. The expression of the prepro-NPY transcript was attenuated after 5 periods of restraint, so that prepro-NPY levels returned almost to normal levels of expression. The expression of prepro-NPY mRNA then increased after 10 days of the restraint paradigm. This apparent biphasic response may represent an attempt to adapt to chronic restraint. The NPY-containing neurons in these nuclei may be stimulated by acute restraint for periods of less than 3 or 4 days. After this acute period, the neuronal network governing various facets of the stress response, that appears to include connections that contain NPY in the SHR, may start to alter their activity in periods of repeated restraint and the resultant response to restraint is attenuated. However, as exposure to restraint continues for a much longer period of time (10 days), the NPY system is activated once again. A future experiment could quantify central prepro-NPY mRNA levels in WKY and SHR strains at time periods of 6, 7, 8 or 9 days of restraint. This restraint protocol would provide a clearer and more detailed description of the changes in prepro-NPY mRNA expression between 5 and 10 sessions of restraint. The cause of the secondary activation of NPY-containing neurons induced by 10 days of restraint is unclear. Furthermore, it is not known whether the changes in prepro-NPY mRNA following restraint are primary mediators of the stress response in SHR or a secondary result of neurochemical changes in other parts of the CNS. Thus, experiments are also required that investigate the function of the NPY system in the SHR during times of chronic restraint and the physiological consequences of any alterations.

### 3.4.6 CONCLUSION

The present chapter utilised standard ISHH techniques that enabled quantification of the transcripts encoding the mRNA of precursors for the neuropeptides enkephalin, dynorphin, GAL and NPY in the CNS of WKY and SHR. Using a single oligonucleotide probe or a combination of probes (proDYN, prepro-GAL and prepro-NPY), markedly different topographic distribution profiles were identified that were specific for each neuropeptide mRNA. Comparison of basal levels of the mRNA for each of the neuropeptides in the CNS of WKY and SHR revealed the presence of significant differences in a number of regions. These significant changes were

specific for each neuropeptide and detected in a variety of regions in the forebrain, pons and medulla oblongata. Regions where some of these changes were localised have been associated with central cardiovascular regulation, such as the RVLN (prepro-ENK, prepro-GAL) and NTS (proDYN), or with other physiological functions, such as the ARC (prepro-NPY), SON (proDYN, prepro-GAL), CPu (proDYN) and cerebellum (prepro-ENK). These data therefore provide future targets for research directed at finding underlying neurochemical alterations that may contribute to the phenotype characteristic of the SHR strain, such as hypertension, hypoalgesia and hyperactivity as compared to WKY (see section 1.4.3).

The activity of these central neuropeptide systems during exposure to acute and chronic restraint was also examined and compared in both strains. As outlined in the pertinent results and discussion sections, acute restraint elicited significant changes in the expression of the mRNA encoding each of these precursors. These changes were neuropeptide-, region- and strain-specific, demonstrating that stressors such as restraint have the capacity to produce alterations in the activity in a wide variety of regions and neurochemical systems in different rat strains. Exposure to repeated restraint often produced a different response when compared to acute restraint, clearly demonstrating the plasticity of each of these neuropeptide modulatory systems. While previous reports have suggested that SHR rats have an impaired ability to respond to stress, the present chapter was able to show that prepro-ENK, proDYN, prepro-GAL and prepro-NPY mRNA expression was dynamically regulated during exposure to acute and chronic restraint, with repeated restraint producing changes in mRNA levels in selected regions that were different to those induced by acute restraint. However, strain-related differences in the restraint-induced response of neurons containing these neuropeptides occurred in particular nuclei implicated in various components of the stress response, suggesting that some of these neurochemical changes may contribute to the altered central, physiological and behavioural stress response previously observed in SHR (see section 1.4.4).

# AUTORADIOGRAPHY



## Autoradiography

aw''to-ra'deog'rə-fe

Noun - the making of a radiograph of an object or tissue by recording on a photographic film the radiation emitted by radioactive material within it.

# CHAPTER 4

## AUTORADIOGRAPHY

### 4.1 INTRODUCTION

The technique of autoradiography provides a visual representation of the tissue distribution of a specific ligand that has been labelled with a radioactive isotope. Receptor autoradiography is simply the focus on the binding of a radioligand to a receptor that is present in central or peripheral tissues. The consequences and downstream biochemical changes that result from the binding of the ligand to the receptor are insignificant, as receptor autoradiography is solely focussed on the amount of receptor binding of the ligand and the interaction between the radioligand and binding site.

Early autoradiography studies sometimes involved *in vivo* injection of the ligand, after which time the brain was removed, sectioned and mounted onto slides with coverslips that had been dipped in photographic emulsion (Kuhar & Yamamura, 1975). Suffice to say this method was limited by the degree of uptake and physicochemical properties of the ligand. Currently, common autoradiographic techniques involve the removal of the brain, sectioning and mounting onto slides prior to incubation with the radioligand. This present method is more economical, the ligand can penetrate the tissues and gain access to receptors, and there is less time between incubation (i.e. receptor binding) and drying of the tissue. This last methodological difference is very important, as it increases the amount of ligand bound to the receptor, and hence maximises signal intensity, by reducing loss of binding due to ligand dissociation.

Autoradiography has been used to describe the central distribution of receptors for neuropeptides such as GAL and the opioids (Skofitsch *et al.*, 1986; Tempel & Zukin, 1987). The discovery of biologically active fragments of the parent peptide, in conjunction with the development of synthetic peptide analogues can uncover different subtypes of a particular receptor. For example, the classical 3 opioid receptor subtypes ( $\mu$ ,  $\delta$  and  $\kappa$ ) have been cloned and extensively characterised throughout the rat CNS (see section 1.3.1). Once discovered,

cloned and purified, the different receptor subtypes can be characterised according to their affinities for the peptide, fragments and analogues. Therefore, an understanding of the binding characteristics is important when designing an autoradiography experiment. In particular, the experimental conditions that are unique for each ligand need to be optimised. The incubation mixture can contain different salts, enzyme inhibitors and unlabelled ligands to act as blocking agents for particular receptors, and preliminary autoradiography experiments are often required to determine the optimal conditions.

The apposition protocol has also undergone development over the years, with different options available for use depending on the aims of the experiment. Researchers can use a microscopic approach, where prefixed tissue sections are dipped directly into emulsion or an emulsion-coated coverslip is glued or fixed directly above the slide-mounted tissue section (e.g. Mansour *et al.*, 1994c). Following development, clusters of silver halide grains exposed by radioisotopic emissions denote the presence of the radioligand, whether it be specific or non-specific tissue binding. Using a light or electron microscope, the presence of silver grains can be visualised, and the anatomical location of the silver grain clusters can be assessed and often assigned to specific cell types. Depending on the resolution, the electron microscope can be used to view receptor distribution at the subcellular level in the perikarya and/or on pre- or post-synaptic specialisations.

Alternatively, following incubation with the radioligand and subsequent drying of the tissue section, the slides can be directly apposed to a large plastic film coated with emulsion or sensitive to the appropriate radiation emitted by the radioligand. This macroscopic approach can provide a detailed map of the distribution of the particular receptor in the CNS or periphery (e.g. Mansour *et al.*, 1993; Mansour *et al.*, 1994b; Mansour *et al.*, 1994c). Concurrent apposition of the appropriate standard microscopes, which consists of a number of tissue pieces that contain a known radioligand concentration, can provide the tools necessary for converting densitometric measurements into relative concentrations of ligand bound to the receptor in the particular region of interest. This facet of macroscopic receptor autoradiography is extremely useful, as it enables quantification of the autoradiographic signal and provides researchers with a valuable tool to measure the effects of experimental manipulations on receptor density.

Receptor autoradiography using the latter approach will be utilised in this chapter. Firstly, the density of opioid ( $\mu$ ,  $\delta$  and  $\kappa$ ) receptors will be compared in the CNS of WKY and SHR rats. As detailed in the ensuing discussion (section 4.4), previous research regarding the distribution

and density of opioid receptors in the hypertensive rat brain has been limited, either anatomically or by non-selective ligands. There is therefore a need for a detailed investigation of central opioid receptor density in SHR, considering that many studies have revealed functional differences in opioid neurochemistry in the brain of this hypertensive rat strain (see section 1.4.2.1).

Similarly, GAL neurochemistry appears to be altered in the CNS of SHR when compared to WKY, as described by previous studies and also in the previous chapter (sections 1.4.2.2 and 3.3.4.1). However, there have been no studies of the distribution of GAL receptors in the CNS of hypertensive rats. Therefore, as an extension of the results in chapter 3 and those outlined in section 1.4.2.2, chapter 4 will compare the density of [<sup>125</sup>I]-GAL binding sites in selected regions of the CNS of SHR and WKY.

Using the same protocol as in chapter 3, rats from both strains will be exposed to a restraint stress paradigm and changes in opioid ( $\mu$ ,  $\delta$  and  $\kappa$ ) and GAL receptor density will be quantified in selected brain regions. While previous studies have demonstrated that the various opioid receptor subtypes in the CNS of normotensive rats are sensitive to different stressors, there have been no autoradiographic investigations of the effects of stress on the central GAL system in WKY. Moreover, no studies have assessed the effects of restraint, or any stressor, on the density of opioid or GAL receptors in the CNS of hypertensive rats.

## 4.2 METHODS

### 4.2.1 RESTRAINT PARADIGM

For the details regarding animals and ethics, refer to sections 2.1 and 2.2. For a general description of the restraint paradigm, refer to section 2.3. Rats from both strains (WKY and SHR) were subjected daily to 60 min restraint stress for 1, 3, 5 or 10 consecutive days (n=3 rats per strain for each restraint period). Rats in the control group (0 days of stress; n=3 per strain) were transferred to the laboratory, remained in their home cage for 1.5 hours (i.e. the same time in the laboratory as the stressed rats) and immediately killed as described below. The protocol

for each day of restraint stress was identical, with rats being exposed to restraint at the same time each day between 10:00h and 12:00h. After the restraint period, each rat was released from the tube and either transferred to its home cage or immediately killed by decapitation. Following decapitation, brains were dissected and removed, frozen over liquid nitrogen and stored at -80°C until further use.

## 4.2.2 PREPARATION OF SLIDE-MOUNTED BRAIN SECTIONS

For the collection of brain sections from both WKY and SHR rats, refer to section 2.5. Sections were thaw-mounted onto gel-chrome alum microscope slides that were prepared according to the protocol described in section 2.5.2 and stored at -80°C until the day of each autoradiography experiment.

## 4.2.3 OPIOID RECEPTOR AUTORADIOGRAPHY

### 4.2.3.1 $\mu$ -Opioid receptors

The Chizzonite method was employed by Prof. B. Jarrott to iodinate FK 33-824 ([D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Met(O)ol<sup>5</sup>]-Enkephalin) using [<sup>125</sup>I]Na and iodogen (Chizzonite *et al.*, 1991). [<sup>125</sup>I]-FK 33-824 has been used as a selective  $\mu$ -opioid receptor ligand in previous studies (Rothman *et al.*, 1987; Cowen *et al.*, 1999). Sodium iodide ([<sup>125</sup>I]Na; 1mCi; 10 $\mu$ l) and 35  $\mu$ l of 0.3M potassium phosphate (pH 7.4) were added to a 1.5ml Eppendorf polypropylene microfuge tube that had been previously coated with 35 $\mu$ g Iodogen. The contents of the microfuge tube were mixed at room temperature for 4 min. A 20 $\mu$ l aliquot of this solution was then combined with FK 33-824 (5 $\mu$ l of a 1 $\mu$ g/ $\mu$ l solution; pH 7.4) and subsequently incubated for 10 min at room temperature. At the end of the incubation, 100 $\mu$ l of 10mM potassium phosphate with 0.1% bovine serum albumin (BSA) was added, mixed and left to stand for 3 min at room temperature. [<sup>125</sup>I]-FK 33-824 was then purified using a pre-washed reverse phase Varian Phenyl cartridge and eluted with a methanol step gradient. As shown in Table 4.1, the fraction that contained the majority of [<sup>125</sup>I]-FK 33-824 was the 40% methanol fraction.

On the day of the autoradiography experiment, tissue sections were removed from  $-80^{\circ}\text{C}$  storage and allowed to warm to room temperature. Slides were then incubated in 50mM Tris-HCl (pH 7.4) containing 0.1% BSA and 0.1nM [ $^{125}\text{I}$ ]-FK 33-824 for 60 min at room temperature (Cowen *et al.*, 1999). Non-specific binding was determined in the presence of 10 $\mu\text{M}$  naloxone. At the conclusion of the incubation period, slides were subjected to 3 X 4 min washes in ice-cold 50mM Tris-HCl and a dip in distilled water. Slides were then dried under a stream of cold air and apposed to X-ray film with standard [ $^{14}\text{C}$ ] microscales for 8 days.

**TABLE 4.1**

FRACTION	DPM
W1	287188
W2	6565
10% MeOH	6068
20% MeOH	6732
30% MeOH	13520
40% MeOH	1318164
50% MeOH	273045
60% MeOH	44722

The elution profile of [ $^{125}\text{I}$ ]-FK 33-824 using an increasing methanol (MeOH) stepwise gradient. W1 and W2 refer to 2ml washes using 10mM potassium phosphate containing 0.1% BSA. Radioactivity, and hence content of [ $^{125}\text{I}$ ]-FK 33-824, was measured using 5 $\mu\text{l}$  aliquots taken from each 2ml fraction and is expressed as DPM.

### 4.2.3.2 $\delta$ -Opioid receptors

The methodology for  $\delta$ -opioid receptor autoradiography was adapted from a number of protocols (Yamamura *et al.*, 1992; Contreras *et al.*, 1993; Drower *et al.*, 1993). Tissue sections were warmed to room temperature and subjected to a 15 min pre-incubation in 50mM Tris-HCl containing 0.1M NaCl (pH 7.4) at room temperature. After this pre-incubation, slides were incubated at 37°C in 50mM Tris-HCl (pH 7.4) that contained 0.1nM [ $^3$ H]-naltrindole, 5mM MgCl<sub>2</sub> and 0.2% BSA for 90 min. Following this, sections were washed in ice-cold 50mM Tris-HCl buffer containing 5mM MgCl<sub>2</sub> and 1% BSA for 4 X 10 min. Following a rapid dip in ice-cold distilled water, slides were dried under a stream of cold air and apposed to tritium-sensitive film in autoradiographic cassettes with standard tritium microscopes for 16 weeks. Non-specific binding was measured in the presence of 10 $\mu$ M naloxone.

### 4.2.3.3 $\kappa$ -Opioid receptors

Tissue sections were removed from -80°C storage, warmed to room temperature and pre-incubated at 37°C for 30 min in 50mM Tris-HCl (pH 7.4). Sections were then incubated at room temperature in 50mM Tris-HCl (pH 7.4) containing 5nM [ $^3$ H]-U69-593 and 0.1% BSA for 90 min (Lahti *et al.*, 1985). Naloxone (10 $\mu$ M) was used to assess non-specific binding. Following the incubation, slides were subjected to 5 X 3 min washes in ice-cold Tris-HCl and a dip in ice-cold distilled water. Slides were then dried under a stream of cold air and apposed to tritium sensitive film with standard tritium microscopes for 16 weeks.

## 4.2.4 GAL RECEPTOR AUTORADIOGRAPHY

Prior to each [ $^{125}$ I]-GAL autoradiography experiment, brain sections were warmed to room temperature after removal from -80°C storage. Sections were subjected to a 15 min preincubation in 50mM Tris-HCl buffer containing 10mM MgCl<sub>2</sub> and 0.5% BSA (pH 7.4). Following the pre-incubation, brain sections were incubated for 60 min in the same buffer containing 0.05nM [ $^{125}$ I]-GAL (rat). Non-specific binding was assessed in the presence of 1 $\mu$ M unlabelled GAL. Sections were subsequently washed in ice-cold Tris buffer (3 X 1 min; 50mM;

pH 7.4), dipped in ice-cold distilled water and dried under a stream of cold air. Slides were then apposed to X-ray film with the appropriate [ $^{14}\text{C}$ ] microscales for 4 days.

#### 4.2.5 PROTOCOL CONSIDERATIONS

Similar to ISHH experiments, autoradiography experiments can vary from day to day. To minimise this experimental variation, autoradiography experiments were conducted using a method similar to that described in section 3.2.3.5. Each autoradiography experiment involved the simultaneous processing (from incubation with the radiolabelled ligand to photographic development of the autoradiograms) of multiple sections from one brain region only (forebrain, pons or medulla) from 30 rats (15 WKY and 15 SHR) for each ligand on the same day. Similar to the ISHH studies in chapter 3, a minimum of 180 sections were processed in each autoradiography experiment. Specifically, this protocol meant that the results component of this autoradiography chapter consisted of 15 separate experiments (i.e. 3 regions X 5 receptor ligands). As with ISHH, this protocol ensured that quantitation of receptor density using autoradiograms from the different rats enabled valid comparisons of the effects of restraint stress on receptor density.

#### 4.2.6 DEVELOPMENT OF FILMS AND PROCESSING OF SECTIONS

When the slide-mounted sections had been apposed to film for the appropriate time, the film was taken out of the cassettes and processed automatically (X-ray films) or manually (tritium-sensitive films) as described in section 2.9.1. The brain sections were then stained and prepared for light microscopy as detailed in section 2.10.

#### 4.2.7 IMAGE ANALYSIS AND STATISTICS

An MCID M4 image analysis system was utilised to analyse all autoradiograms generated by the autoradiography experiments (section 2.12). Results were expressed as mean  $\pm$  S.E.M., and the statistical program, GraphPad Prism®, was used for all statistical analyses. For each region

that was analysed, three separate statistical tests were employed which have been described in section 3.2.5. Briefly, these are:

BETWEEN STRAIN COMPARISON – BASAL DIFFERENCES IN RECEPTOR DENSITY (unpaired student's *t*-test).

WITHIN STRAIN COMPARISON – EFFECT OF RESTRAINT (one way ANOVA).

BETWEEN STRAIN COMPARISON – EFFECT OF RESTRAINT (two way ANOVA).

In all cases, a level of  $P < 0.05$  was considered to be statistically significant.

## 4.2.8 MATERIALS AND ABBREVIATIONS

For all materials used in this chapter, refer to sections 2.14.1 and 2.14.3. For all abbreviations, refer to page xvii.

## 4.3 RESULTS

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### 4.3.1 $\mu$ -OPIOID RECEPTOR AUTORADIOGRAPHY

#### 4.3.1.1 *Strain comparison*

Using [ $^{125}$ I]-FK 33-824,  $\mu$ -opioid binding was visualised in multiple regions throughout selected levels of the CNS of both normotensive (WKY) and hypertensive (SHR) rats (Figure 4.1). As demonstrated in Figure 4.2, the thalamus of both strains contained relatively dense populations of  $\mu$ -opioid receptors, with one of the highest density of  $\mu$  receptors located in the Hab. Moderate to high densities of  $\mu$ -opioid binding sites were visualised in the cortex (Par, RS, Fr) and the BM and BL, two subregions of the amygdala. In the pons and medulla,  $\mu$ -opioid receptor populations were not as widely distributed, with low to moderate densities of [ $^{125}$ I]-FK

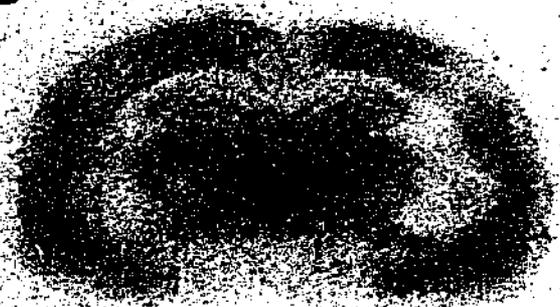
**FIGURE 4.1**

Autoradiographic images of [<sup>125</sup>I]-FK 33-824 binding sites in the forebrain (A-D), pons (E, F) and medulla oblongata (G, H) of normotensive (WKY; A, C, E, G) and hypertensive (SHR; B, D, F, H) rat brain. Panels C and D represent the non-specific binding of [<sup>125</sup>I]-FK 33-824 in the presence of 10μM naloxone in the forebrain of WKY and SHR respectively. Scale bar represents 2.42mm (A-D), 2.28mm (E, F) or 1.33mm (G, H).

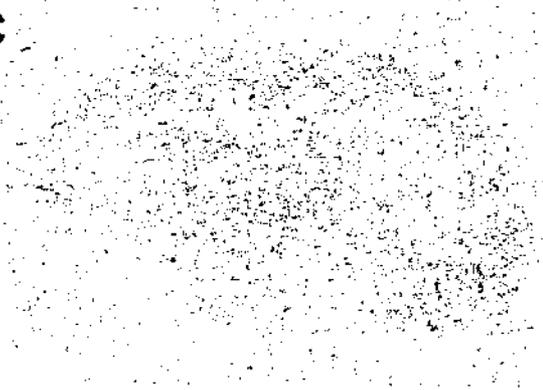
**A**



**B**



**C**



**D**



**E**



**F**



**G**

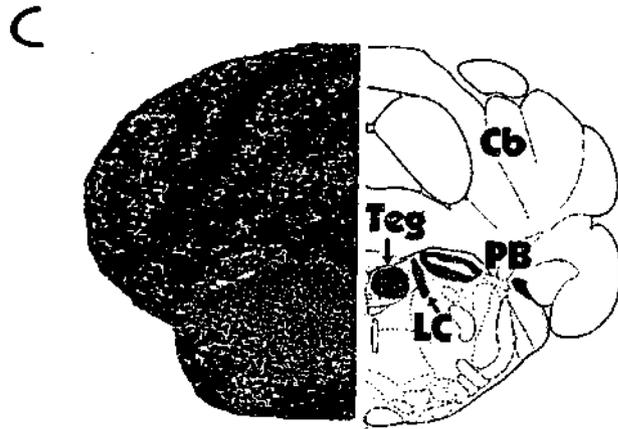
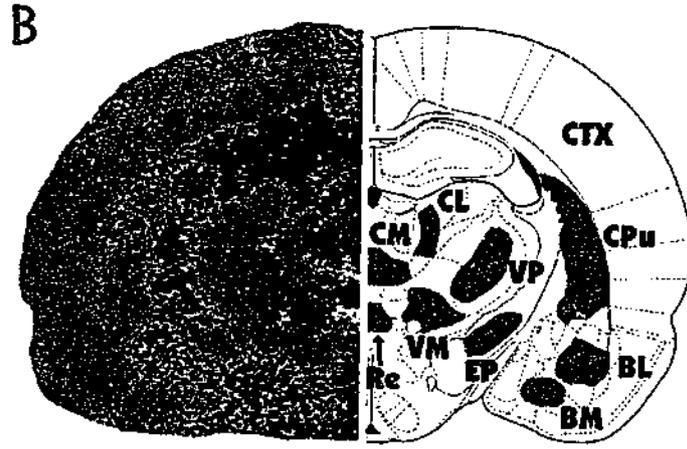
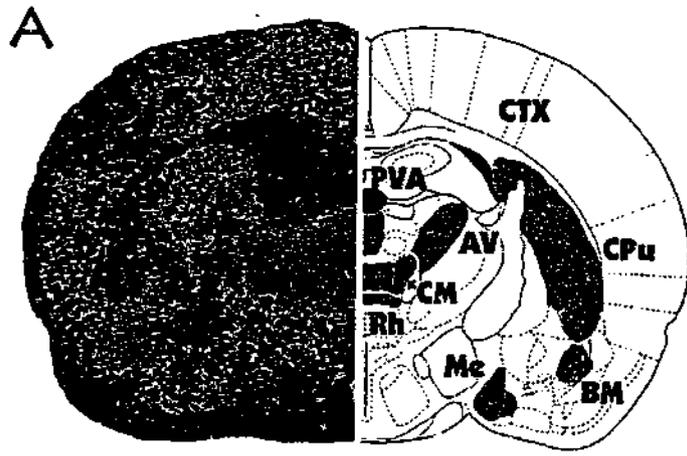


**H**



**FIGURE 4.2**

Representative pseudocolour autoradiograms and corresponding brain maps demonstrating the distribution of [ $^{125}$ I]-FK 33-824 binding sites at four levels of the rat CNS (A – forebrain: bregma ~ -1.8mm; B – forebrain: bregma ~ -2.8mm; C – pons: bregma ~ -9.7mm; D – medulla oblongata: bregma ~ -13.3mm). For abbreviations, refer to page xvii. Scale bar represents 1.88mm (A and B), 1.45mm (C) and 1.08mm (D).



33-824 binding sites quantified in the PB, Teg, cerebellum, Cu, gracile nucleus (Gr) and NAmb.  $\mu$ -Opioid receptors were also visualised in the NTS of both strains, with receptors present at the commissural and medial (subpostremal and bilateral) levels. Figure 4.2D shows that the highest density of [ $^{125}$ I]-FK 33-824 binding sites in the medulla oblongata were detected in the NTS. However, when [ $^{125}$ I]-FK 33-824 binding site density was compared between the NTS and forebrain regions (Table 4.2), it can be clearly seen that regions such as the thalamus and BL contain a greater density of  $\mu$ -opioid receptors. Regions where  $\mu$ -opioid receptor populations were observed but not quantified included the hippocampus, amygdala (Me), CPu, LHy and LC (Figure 4.2 and Table 4.2). Interestingly,  $\mu$ -opioid receptors were not detected in moderate to high densities in the majority of hypothalamic nuclei, including the PVN (Figure 4.1 and 4.2).

The distribution of  $\mu$ -opioid receptors was similar in the CNS of WKY and SHR at the levels of the neuraxis that were examined (Figure 4.1). Comparison of the density of [ $^{125}$ I]-FK 33-824 binding sites in individual central nuclei in WKY and SHR revealed significantly altered levels of  $\mu$ -opioid receptors existed in a number of regions (Figure 4.3). The BL of SHR contained significantly increased (+32%;  $P < 0.05$ ; student's unpaired  $t$ -test) levels of the  $\mu$ -opioid receptor compared to WKY. Although cortical populations of  $\mu$ -opioid receptors were visualised in a number of subregions (RS, Fr, Par), the density of [ $^{125}$ I]-FK 33-824 binding sites was quantified in the cortex as a single structure, with a significantly reduced density of [ $^{125}$ I]-FK 33-824 binding sites found in SHR compared with WKY (-15%;  $P < 0.01$ ; student's unpaired  $t$ -test). The thalamus was also found to contain a significantly lower density of  $\mu$ -opioid receptors when compared to WKY (-15%;  $P < 0.05$ ; student's unpaired  $t$ -test). The density of  $\mu$ -opioid receptors was quantified in the cerebellum at the level of the pons, and comparison between strains revealed that SHR had a significantly increased level of [ $^{125}$ I]-FK 33-824 binding sites compared to WKY (+19%;  $P < 0.05$ ; student's unpaired  $t$ -test). As described earlier,  $\mu$ -opioid receptor density was quantified separately in the commissural (caudal) and medial (rostral) subregions of the NTS in each strain. In both strains, the medial NTS contained a higher density of  $\mu$ -opioid receptors compared to the commissural subnucleus, but only in the SHR was this difference significant (+51%;  $P < 0.01$ ; paired student's  $t$ -test) (Figure 4.3). Moreover, there were no significant differences in [ $^{125}$ I]-FK 33-824 binding site density between strains at either level of the NTS.

**FIGURE 4.3**

Comparison of basal (no stress) [ $^{125}$ I]-FK 33-824 binding site density in the CNS of normotensive (WKY) and hypertensive (SHR) rat strains (n=3 per strain). Density of [ $^{125}$ I]-FK 33-824 binding sites is expressed as the % change in SHR compared to WKY. For abbreviations, see page xvii.

\*:  $P < 0.05$ ; unpaired student's *t*-test.

# % CHANGE IN SHR COMPARED TO WKY

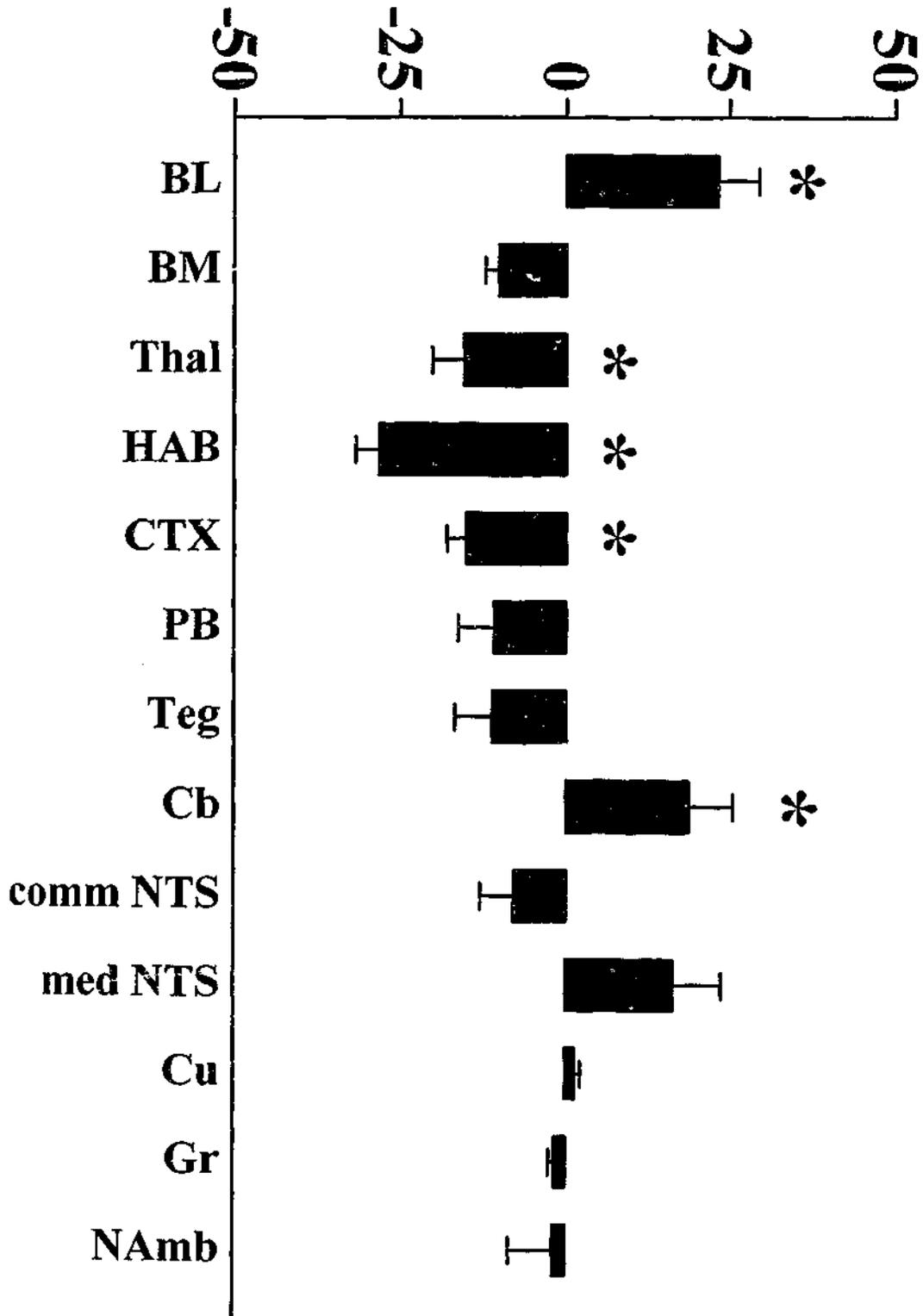


TABLE 4.2

REGION	RELATIVE DENSITY OF [ <sup>125</sup> I]-FK 33-824 BINDING SITES	
	WKY	SHR
<b>FOREBRAIN</b>		
BL	+++	+++
BM	++	++
Me	+	+
Thal	+++	+++
AV	+++	+++
CM	+++	+++
Hab	+++	+++
PVA	+++	+++
Rh	+++	+++
CPu	++	++
CTX	+	+
<b>PONS</b>		
LC	+	+
PB	+	+
Teg	+	+
Cb	+	+
<b>MEDULLA</b>		
comm NTS	++	++
med NTS	++	++
Cu	+	+
Gr	+	+
NAmb	++	++
sp5	+	+

Relative distribution of [<sup>125</sup>I]-FK 33-824 binding sites in the CNS of WKY and SHR rats. The density of [<sup>125</sup>I]-FK 33-824 binding sites was assessed as follows: +++ - dense (> 4 DPM/mm<sup>2</sup>); ++ - moderate (2 DPM/mm<sup>2</sup> to 4 DPM/mm<sup>2</sup>) and + - light (< 2 DPM/mm<sup>2</sup>). For abbreviations, see page xvii.

### 4.3.1.2 Restraint stress and [<sup>125</sup>I]-FK 33-824 binding

Restraint stress caused a region- and strain-specific regulation of [<sup>125</sup>I]-FK 33-824 binding (Figure 4.4). In the BL of WKY, neither acute nor chronic restraint had a significant effect on  $\mu$ -opioid receptor density. In contrast, acute (1 session ( $P < 0.05$ ) and 3 sessions ( $P < 0.01$ )) and chronic (10 sessions ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test)) restraint induced significant decreases of up to -28% in the BL of SHR (Figure 4.4A). Furthermore, a significant interaction between strain and number of restraint sessions was observed in the BL ( $F(4,20) = 4.37$ ;  $P < 0.01$ ; two way ANOVA). This effect was restricted to the BL subregion of the amygdala, with no alterations in  $\mu$ -opioid receptor density observed during the restraint paradigm in the BM of either strain (Figure 4.4B).

$\mu$ -Opioid receptors in the cortex of both strains were sensitive to perturbation by restraint stress (Figure 4.4D). In WKY, acute restraint produced a significant reduction (-19%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test) in  $\mu$ -opioid receptor density, with subsequent exposure to restraint having no effect on the levels of the  $\mu$ -opioid receptor in the cortex. However, a clearly different temporal response was observed in the cortex of SHR. While acute restraint did not elicit any changes in  $\mu$ -opioid receptor density, a significant decrease of -16% was detected after 10 periods of restraint stress in the SHR cortex ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). Subsequent two way ANOVA analysis demonstrated that the temporal response to the restraint paradigm was significantly different between strains ( $F(4,20) = 3.01$ ;  $P < 0.05$ ; two way ANOVA). In addition, there were regions such as the thalamus and cerebellum in both strains that did not exhibit significant changes in [<sup>125</sup>I]-FK 33-824 binding site density during the restraint paradigm (Figure 4.4C and 4.4F).

In the PB, which was not separated into medial and lateral subregions, a significant interaction between strain and restraint paradigm was also found ( $F(4,20) = 3.02$ ;  $P < 0.05$ ; two way ANOVA). In the PB of WKY, acute restraint had no effect on  $\mu$ -opioid receptor density, while 10 restraint periods produced a significantly elevated density of [<sup>125</sup>I]-FK 33-824 binding sites (+58%;  $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test) (Figure 4.4E). In contrast, hypertensive rats did not exhibit any changes in  $\mu$ -opioid receptor density in the PB at any time during the restraint paradigm.

[<sup>125</sup>I]-FK 33-824 binding site density in both the commissural and medial NTS of SHR was not altered by either acute or chronic restraint (Figure 4.4H). Similarly, the medial NTS of

**FIGURE 4.4**

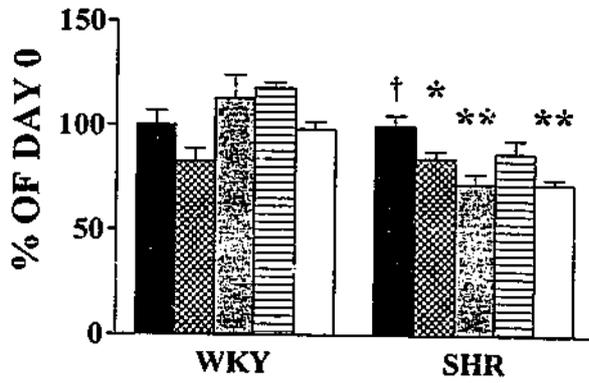
The effect of acute and chronic restraint stress on the density of [<sup>125</sup>I]-FK 33-824 binding sites in selected forebrain regions of normotensive (WKY) and hypertensive (SHR) rat CNS. Rats were exposed to a 60 min period of restraint stress for 0 (control; filled black columns), 1 (hatched columns), 3 (filled grey columns), 5 (horizontal lined columns) or 10 (open columns) consecutive days (n=3 rats per group per strain). Brain regions represented are: A – BL; B – BM; C – Thal; D – CTX; E – PB; F – Cb; G – med NTS and H – comm NTS. Results are expressed as a percentage of day 0 (control) levels ± S.E.M. For abbreviations, refer to page xvii.

\*:  $P < 0.05$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

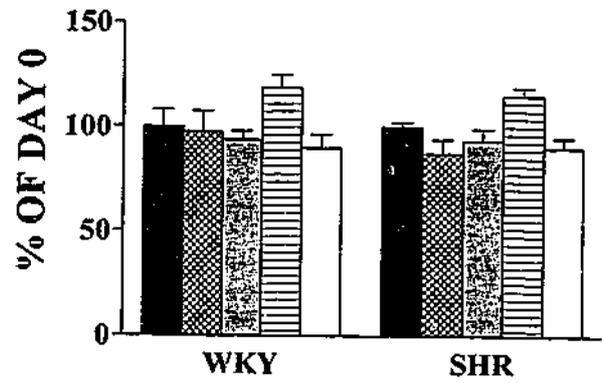
\*\* :  $P < 0.01$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

†:  $P < 0.05$  when comparing the temporal response to restraint in SHR with WKY; two way ANOVA.

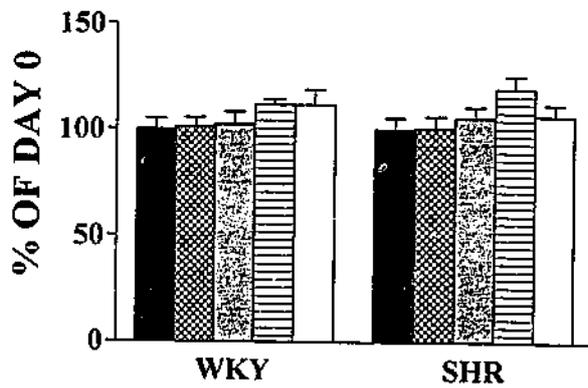
### A - BL



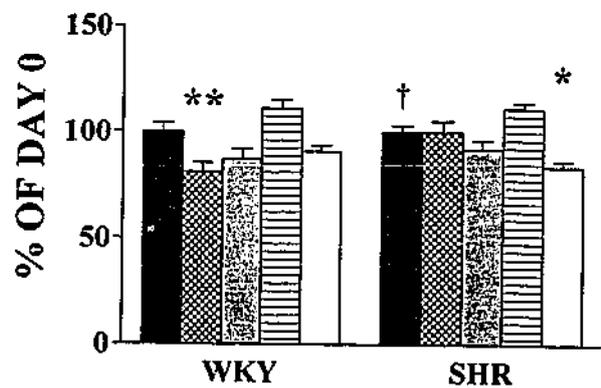
### B - BM



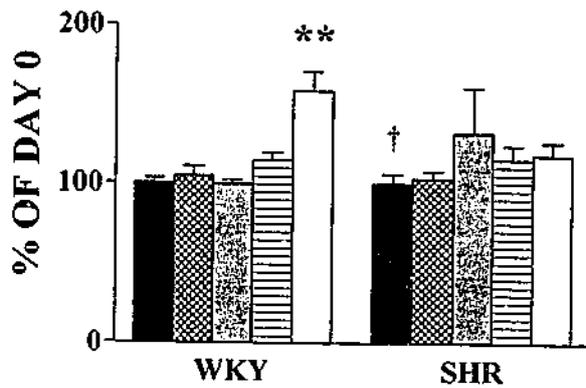
### C - Thal



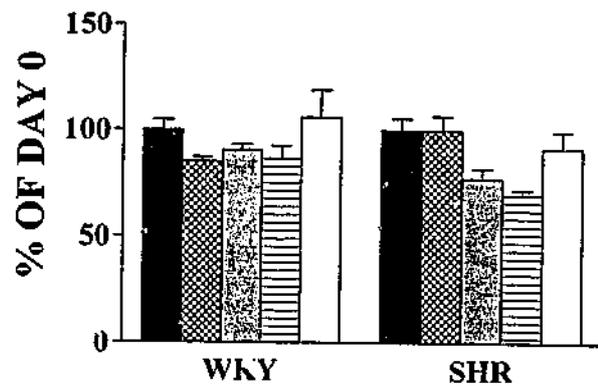
### D - CTX



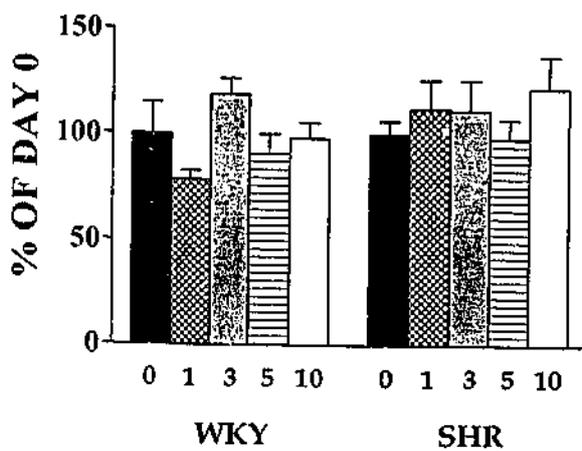
### E - PB



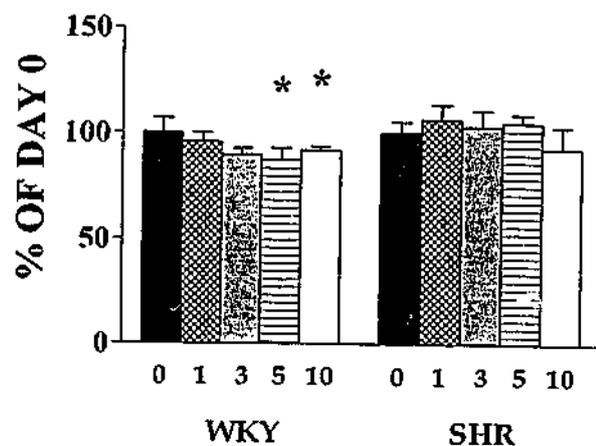
### F - Cb



### G - med NTS



### H - comm NTS



WKY did not exhibit any changes in  $\mu$ -opioid receptor density during the restraint paradigm. However, a steady decrease in  $\mu$ -opioid receptor density was observed in the commissural NTS of WKY rats exposed to restraint. Non-significant reductions were found after 1 and 3 periods of restraint, while 5 (-13%;  $P < 0.05$ ) and 10 sessions of restraint stress (-8%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test) produced a significant attenuation of  $\mu$ -opioid receptor density in the commissural NTS of WKY (Figure 4.4G). Comparison of the temporal response to restraint exhibited by the commissural NTS revealed that there was no significant interaction between strain and the restraint paradigm.

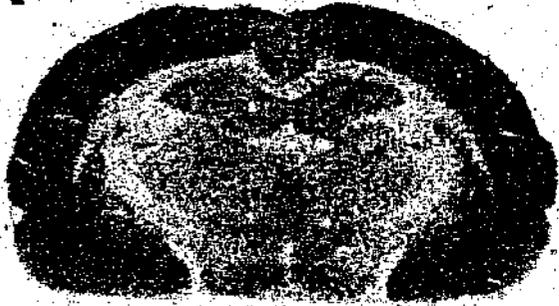
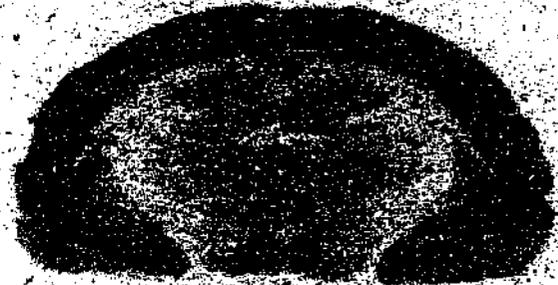
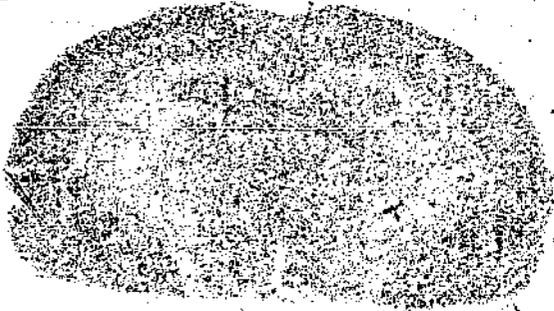
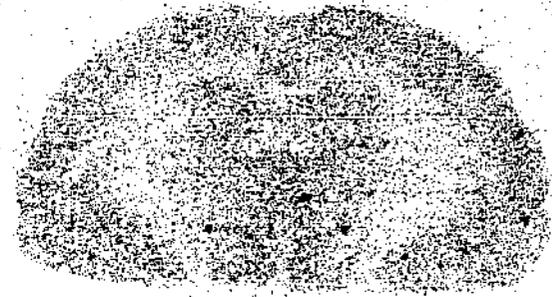
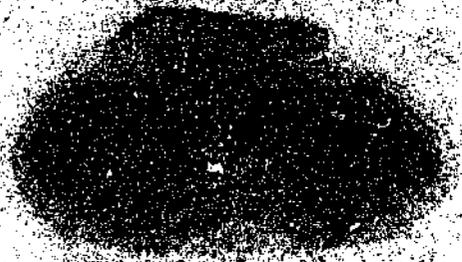
### 4.3.2 $\delta$ -OPIOID RECEPTOR AUTORADIOGRAPHY

#### 4.3.2.1 *Strain comparison*

The radiolabelled  $\delta$ -opioid receptor antagonist [ $^3$ H]-naltrindole was used to visualise the central distribution of  $\delta$ -opioid receptors in both normotensive and hypertensive rats. In both strains, a similar distribution profile was observed, with [ $^3$ H]-naltrindole binding sites observed throughout the forebrain, pons and medulla (Figure 4.5). In the forebrain,  $\delta$ -opioid receptors were detected in moderate to high density in the cortex, CPu, amygdala (BL, BM and Me) and DEn (Table 4.3). Regions such as the hippocampus, st, thalamus, hypothalamus (VMH, LHy) and additional amygdaloid regions were found to contain low densities of [ $^3$ H]-naltrindole binding sites. In the pons and medulla, the density of  $\delta$ -opioid receptors was highest in the NTS, CVLM, XII and Sp5C, with populations of  $\delta$ -opioid receptors also detected in the LC, Teg, cerebellum, IOC and reticular fields (Figure 4.6).

**FIGURE 4.5**

Autoradiographic images of [<sup>3</sup>H]-naltrindole binding site density in the forebrain (A-D), pons (E, F) and medulla oblongata (G, H) of normotensive (WKY; A, C, E, G) and hypertensive (SHR; B, D, F, H) rat brain. Panels C and D represent the non-specific binding of [<sup>3</sup>H]-naltrindole in the presence of 10 $\mu$ M naloxone in the forebrain of WKY and SHR respectively. Scale bar represents 2.42mm (A-D), 2.28mm (E, F) or 1.33mm (G, H).

**A****B****C****D****E****F****G****H**

**FIGURE 4.6**

Representative pseudocolour autoradiograms and corresponding brain maps demonstrating the distribution of [<sup>3</sup>H]-naltrindole binding sites at four levels of the rat CNS (A – forebrain: bregma ~ -1.8mm; B – forebrain: bregma ~ -2.8mm; C – pons: bregma ~ -9.7mm; D – medulla oblongata: bregma ~ -14.1mm). For abbreviations, refer to page xvii. Scale bar represents 1.88mm (A and B), 1.45mm (C) and 1.08mm (D).

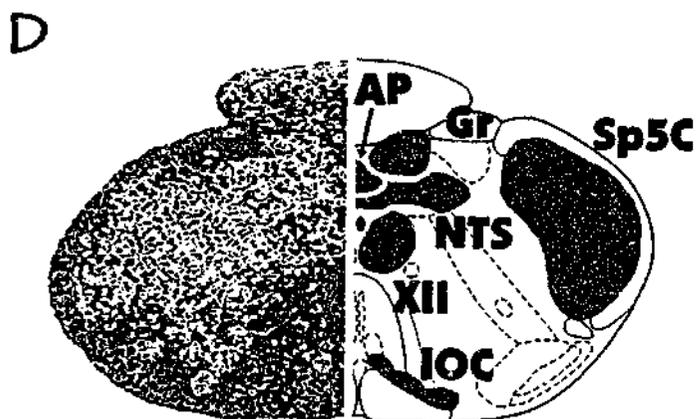
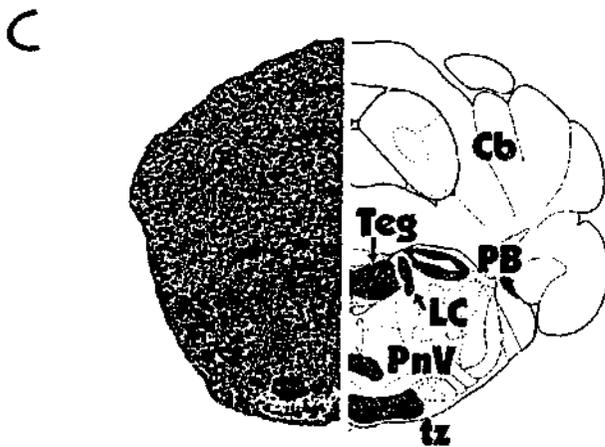
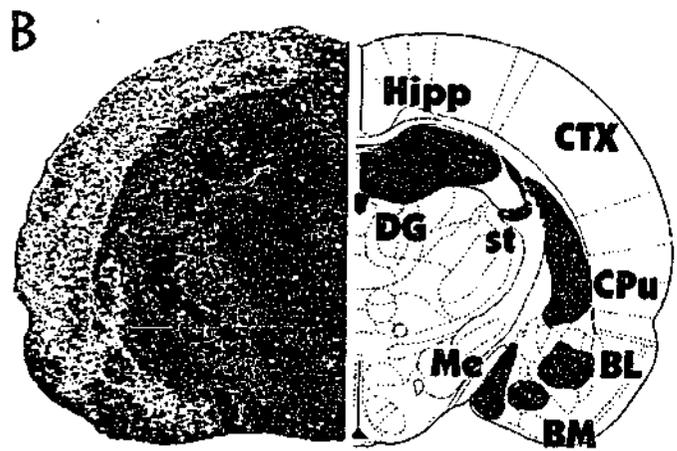
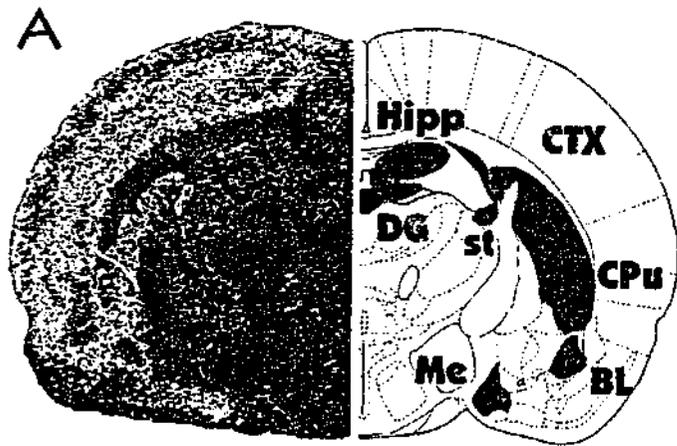


TABLE 4.3

REGION	RELATIVE DENSITY OF [ <sup>3</sup> H]-NALTRINDOLE BINDING SITES	
	WKY	SHR
<b>FOREBRAIN</b>		
VMH	++	++
BL	+++	+++
BM	+++	+++
Me	+++	++
CPu	+++	+++
Hipp	++	+
CTX	+++	++
<b>PONS</b>		
LC	+	+
Teg	+	+
PB	+	+
PnV	+	+
ml	+	+
tz	++	++
Cb	+	+
Ent	+	+
<b>MEDULLA</b>		
med NTS	++	+
comm NTS	++	+
AP	++	+
Gr	++	+
ECu	++	+
DMX	++	+
XII	++	+
CVLM	++	+
IOC	++	+
Sp5C	++	+

Relative distribution of [<sup>3</sup>H]-naltrindole binding sites in the CNS of WKY and SHR rats. The density of [<sup>3</sup>H]-naltrindole binding sites was assessed as follows: +++ - dense (> 25 DPM/mm<sup>2</sup>); ++ - moderate (10 DPM/mm<sup>2</sup> to 25 DPM/mm<sup>2</sup>) and + - light (< 10 DPM/mm<sup>2</sup>). For abbreviations, see page xvii.

Following quantification of the density of  $\delta$ -opioid receptors in a variety of nuclei in the CNS of both WKY and SHR, statistical comparison between strains revealed that the SHR generally contained a lower density of [ $^3$ H]-naltrindole binding sites in the majority of central regions (Figure 4.7). The amygdala of SHR was found to contain a significantly reduced density of  $\delta$ -opioid receptors, with the largest difference of -37% occurring in the BL subregion (BL ( $P < 0.01$ ), BM ( $P < 0.05$ ) and Me ( $P < 0.05$ ); unpaired student's  $t$ -test). In the hippocampus, the density of  $\delta$ -opioid receptors was -46% lower in SHR than WKY ( $P < 0.01$ ), while in the CPu, a significantly reduced level of  $\delta$ -opioid receptors was also observed in the SHR strain compared to WKY (-19%;  $P < 0.01$ ; unpaired student's  $t$ -test).

In both strains, similar densities of  $\delta$ -opioid receptors were detected in all pontine nuclei (Figure 4.7). In contrast,  $\delta$ -opioid receptor density was significantly reduced in all medullary nuclei in SHR compared to WKY. In the CVLM of SHR, a significantly decreased (-44%) level of [ $^3$ H]-naltrindole binding sites was detected ( $P < 0.01$ ; unpaired student's  $t$ -test). The largest difference was detected in the medial (rostral and subpostremal) NTS (-65%;  $P < 0.01$ ), while the commissural NTS of SHR also contained a significantly reduced level of  $\delta$ -opioid receptors when compared to WKY (-47%;  $P < 0.01$ ; unpaired student's  $t$ -test) (Figure 4.7). Within strains, the density of  $\delta$ -opioid receptors was compared between the commissural and medial NTS to ascertain whether any differences existed between subregions of the NTS. In WKY, the medial NTS was found to contain a significantly higher density of  $\delta$ -opioid receptors than the commissural NTS ( $P < 0.05$ ; paired student's  $t$ -test). In contrast to the WKY strain, the medial and commissural portions of the NTS in SHR contained similar levels of [ $^3$ H]-naltrindole binding sites.

#### 4.3.2.2 Restraint stress and [ $^3$ H]-naltrindole binding

In addition to a comparison of basal levels of  $\delta$ -opioid receptor density in the CNS of WKY and SHR, the present study also investigated the effects of acute and chronic restraint stress on  $\delta$ -opioid receptor binding sites in these strains. Restraint-induced alterations in  $\delta$ -opioid receptor density were observed in both strains, and as outlined below and in Figure 4.8, these changes were both region- and strain-specific. In all subregions of the amygdala in SHR, neither acute nor chronic restraint had a significant effect on  $\delta$ -opioid receptor density. Similarly, acute and chronic restraint did not elicit any changes in [ $^3$ H]-naltrindole binding site density in the BL and

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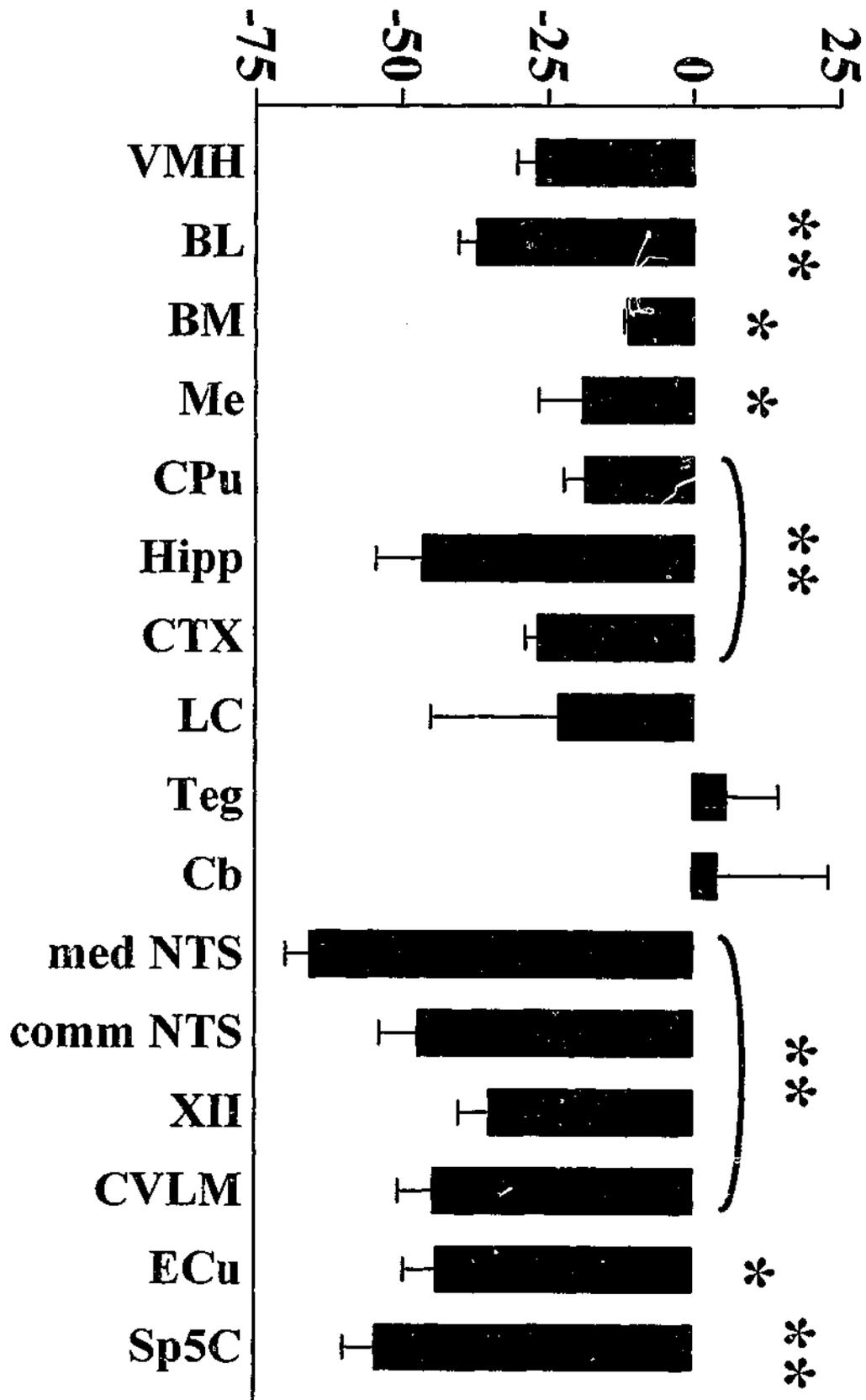
**FIGURE 4.7**

Comparison of basal (no stress) [<sup>3</sup>H]-naltrindole binding site density in the CNS of normotensive (WKY) and hypertensive (SHR) rat strains (n=3 per strain). Density of [<sup>3</sup>H]-naltrindole binding sites is expressed as the % change in SHR compared to WKY. For abbreviations, see page xvii.

\*:  $P < 0.05$ ; unpaired student's *t*-test.

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# % CHANGE IN SHR COMPARED TO WKY



**FIGURE 4.8**

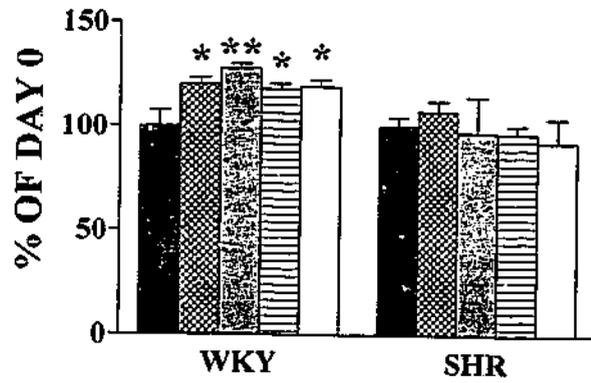
The effect of acute and chronic restraint stress on the density of [<sup>3</sup>H]-naltrindole binding sites in selected regions of normotensive (WKY) and hypertensive (SHR) rat CNS. Rats were exposed to a 60 min period of restraint stress for 0 (control; filled black columns), 1 (hatched columns), 3 (filled grey columns), 5 (horizontal lined columns) or 10 (open columns) consecutive days (n=3 rats per group per strain). Brain regions represented are: A – VMH; B – BM; C – CPu; D – CTX; E – LC; F – CVLM; G – med NTS and H – comm NTS. Results are expressed as a percentage of day 0 (control) levels ± S.E.M. For abbreviations, refer to page xvii.

\*:  $P < 0.05$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

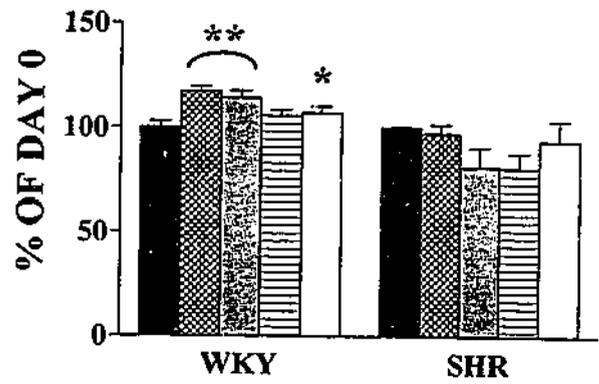
\*\*:  $P < 0.01$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

†:  $P < 0.05$  when comparing the temporal response to restraint in SHR with WKY; two way ANOVA.

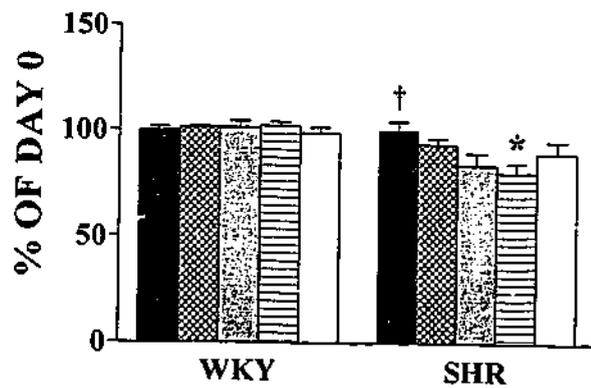
### A - VMH



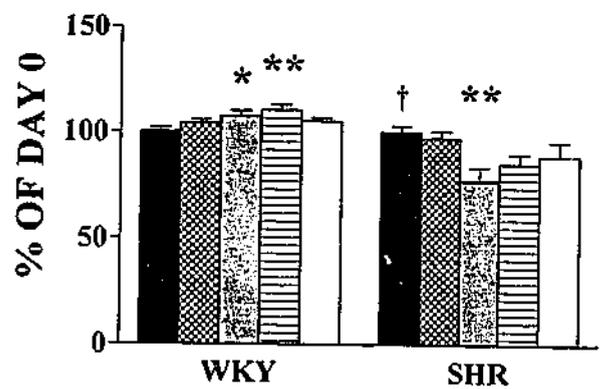
### B - BM



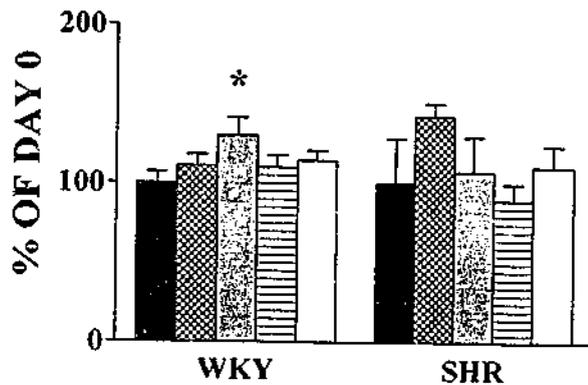
### C - CPu



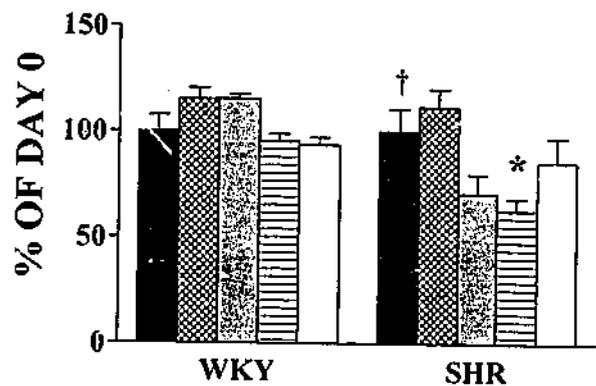
### D - CTX



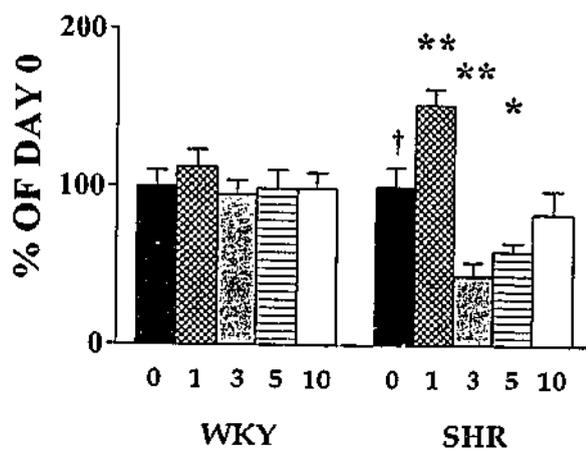
### E - LC



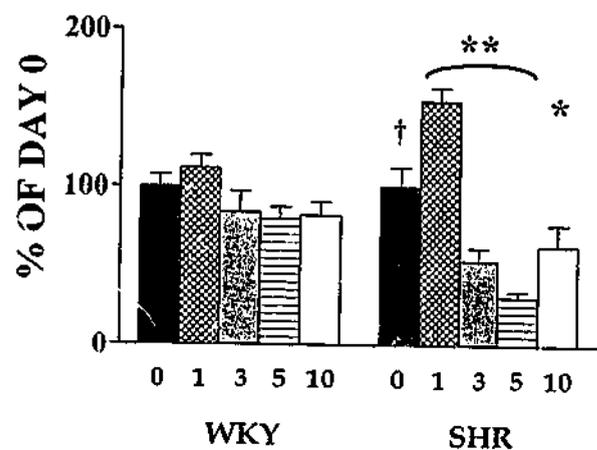
### F - CVLM



### G - med NTS



### H - comm NTS



Me of WKY. However, as shown in Figure 4.8B, acute restraint produced a significant elevation of +17% in  $\delta$ -opioid receptor density in the BM of WKY ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test). Three periods of restraint produced a significantly increased level of  $\delta$ -opioid receptors in the BM of WKY (+14%;  $P < 0.01$ ), while a smaller, but significant increase was also observed after 10 days of the restraint paradigm in this amygdaloid subnucleus (+7%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). Although the temporal response to restraint exhibited by the BM appears to differ markedly between strains (see Figure 4.8B), statistical comparison using a two way ANOVA did not reveal any significant interaction between strain and the restraint paradigm ( $F(4,20) = 1.77$ ;  $P > 0.05$ ; two way ANOVA).

The population of  $\delta$ -opioid receptors in the VMH of WKY was sensitive to perturbation by both acute and chronic restraint (Figure 4.8A). A significant increase in  $\delta$ -opioid receptor density was observed after each exposure to restraint, with the largest elevation of +28% detected after 3 periods of restraint ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test). The temporal response profile demonstrates that after 10 sessions of restraint, the density of  $\delta$ -opioid receptors in the VMH of WKY was still elevated and does not appear to be returning to basal density values. In the VMH of SHR, acute and chronic restraint had no significant effect on the density of [ $^3$ H]-naltrindole binding sites.

In the CPu of WKY, no changes in the density of  $\delta$ -opioid receptors were detected following any period of exposure to restraint (Figure 4.8C). In contrast, 5 sessions of restraint induced a significant decrease in  $\delta$ -opioid receptor density in the CPu of SHR (-20%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). The difference in the temporal response of the CPu to restraint between strains was further supported by the results of a two way ANOVA, where a significant interaction was found between strain and number of periods of restraint ( $F(4,20) = 2.61$ ;  $P < 0.05$ ; two way ANOVA).

The temporal response of the cortical  $\delta$ -opioid receptor system to restraint stress was found to be significantly different between WKY and SHR ( $F(4,20) = 5.25$ ;  $P < 0.001$ ; two way ANOVA). In WKY, acute restraint did not produce any alterations in [ $^3$ H]-naltrindole binding site density, but continued exposure to the restraint paradigm elicited significant increases in  $\delta$ -opioid receptor density. These significant changes were detected after 3 sessions (+7%;  $P < 0.05$ ) and 5 sessions (+10%;  $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test) of the restraint paradigm, with a non-significant increase observed after 10 days of the restraint paradigm. In SHR, a markedly different response profile was observed in the cortex. The

restraint paradigm elicited a bell-shaped response profile, with a significant decrease in the density of  $\delta$ -opioid receptors detected after 3 periods of restraint (-23%;  $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test). Following this nadir after 3 sessions of restraint,  $\delta$ -opioid receptor density in the cortex of SHR gradually returned to basal levels after subsequent exposure to restraint.

As demonstrated in Figure 4.8E, 3 periods of restraint produced a significant increase of +30% in  $\delta$ -opioid receptor density in the LC of WKY ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). Smaller non-significant increases in  $\delta$ -opioid receptor density were observed in the LC of WKY after 5 and 10 days of the restraint paradigm. In the LC of SHR, although the density of  $\delta$ -opioid receptors was quite varied during the restraint stress paradigm, neither acute nor chronic restraint stress had a significant effect on  $\delta$ -opioid receptor levels.

Despite a lack of changes in  $\delta$ -opioid receptor density in the NTS of WKY following exposure to the restraint paradigm, both the medial and commissural subregions of the NTS in SHR exhibited a biphasic response profile in response to restraint (Figure 4.8G and 4.8H). Acute restraint (1 session) elicited a significant increase of approximately +50% in both regions of the NTS in SHR (both  $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test). A significant reduction of almost -50% in the levels of  $\delta$ -opioid receptors was then observed after 3 periods of restraint (both  $P < 0.01$ ), while subsequent exposure to restraint produced smaller decreases in [ $^3$ H]-naltrindole binding site density in the medial and commissural NTS. Further statistical comparison revealed a significantly different temporal response to the restraint paradigm between the WKY and SHR strains in both the medial ( $F(4,20) = 6.15$ ;  $P < 0.001$ ) and commissural NTS ( $F(4,20) = 6.56$ ;  $P < 0.001$ ; two way ANOVA).

A significant interaction between the restraint paradigm and strain was also detected following analysis of restraint-induced levels of  $\delta$ -opioid receptor density in the CVLM of WKY and SHR ( $P < 0.01$ ; two way ANOVA). While there were no significant changes in [ $^3$ H]-naltrindole binding site density in the CVLM of WKY after any period of restraint, 5 days of the restraint paradigm induced a significant reduction of -37% in the CVLM of SHR ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test).

### 4.3.3 $\kappa$ -OPIOID RECEPTOR AUTORADIOGRAPHY

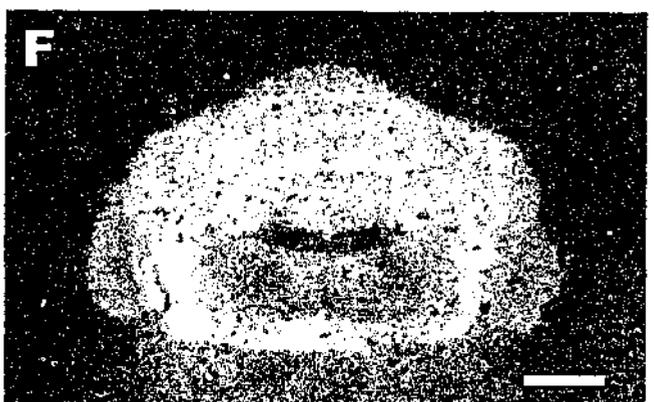
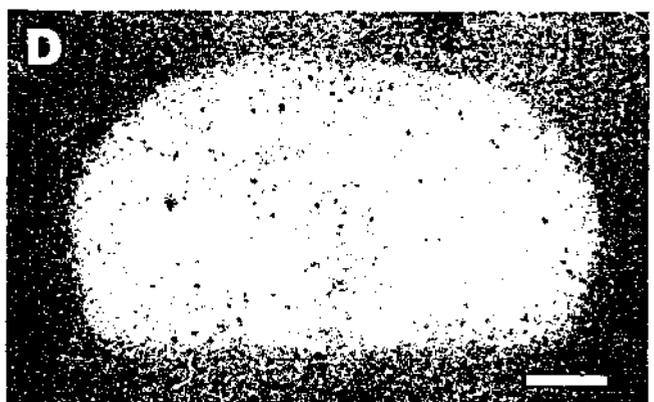
#### 4.3.3.1 *Strain comparison*

$\kappa$ -Opioid receptor binding was detected throughout the CNS of both WKY and SHR rats using the tritiated ligand, [ $^3$ H]-U69-593 (Figure 4.9). Moderate to dense populations of [ $^3$ H]-U69-593 binding sites were visualised primarily in the forebrain in regions such as the PVN, thalamus (PVA, ZI), st, Me, amygdalostriatal transition zone (AM/Str), CPu and Par (Figure 4.10).  $\kappa$ -Opioid receptors were also detected throughout the amygdala, hypothalamus, thalamus and cortex. In the pons, the distribution of  $\kappa$ -opioid receptors was much more restricted, with the densest population of receptors clearly localised in the Teg. Lower levels of  $\kappa$ -opioid receptors were also visualised in the cerebellum and reticular fields. The NTS contained the highest level of [ $^3$ H]-U69-593 binding sites in the medulla while the IOC, Sp5C and reticular fields also contained  $\kappa$ -opioid receptors (Table 4.4). Similar to section 4.3.2.1, the NTS was subdivided into the medial and commissural portions. Despite a trend towards an increased level of  $\kappa$ -opioid receptors in the medial NTS compared to the commissural NTS in both strains, no significant difference was found between these subregions of the NTS in either strain.

Comparison of the density of [ $^3$ H]-U69-593 binding sites throughout selected regions of the CNS of WKY and SHR revealed a global elevation in  $\kappa$ -opioid receptor density in the CNS of SHR rats compared to their normotensive controls. Moreover, in the forebrain of SHR,  $\kappa$ -opioid receptor density was almost twice as high than in the corresponding region in WKY. The largest differences were detected in the st (+163%), DEn (+64%) and Par (+46%; all  $P < 0.01$ ; unpaired student's  $t$ -test) of SHR compared to WKY (Figure 4.11). As seen in Figure 4.11, regions such as the PVN ( $P < 0.01$ ) and Me ( $P < 0.05$ ) also contained a significantly increased level of  $\kappa$ -opioid receptors in SHR compared to WKY (unpaired student's  $t$ -test).

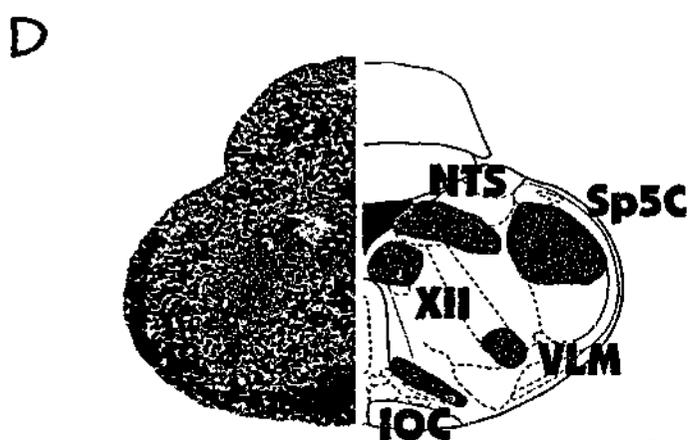
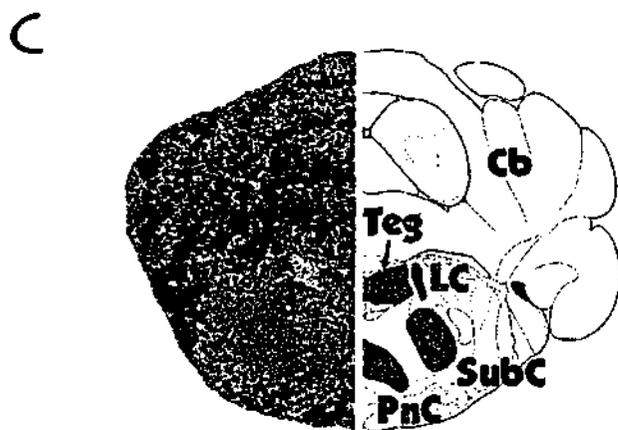
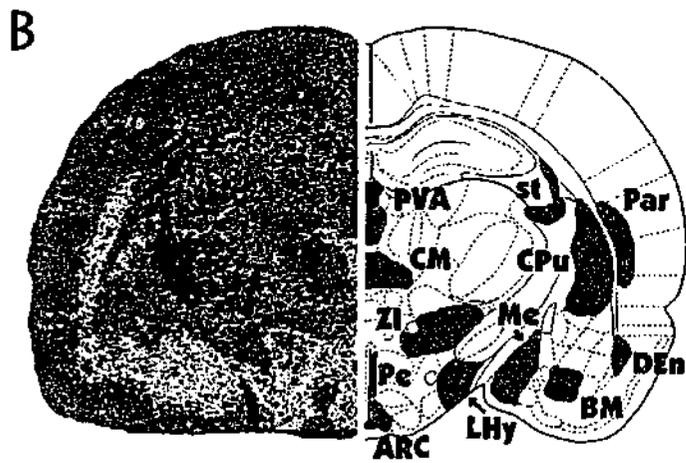
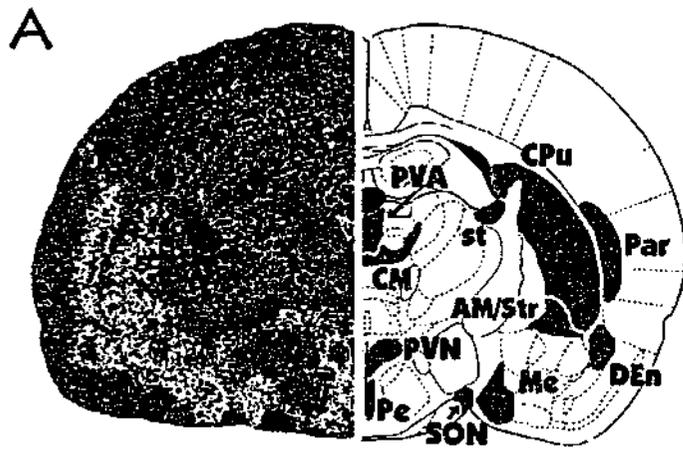
**FIGURE 4.9**

Autoradiographic images of [<sup>3</sup>H]-U69-593 binding sites in the forebrain (A-D), pons (E, F) and medulla oblongata (G, H) of normotensive (WKY; A, C, E, G) and hypertensive (SHR; B, D, F, H) rat brain. Panels C and D represent the non-specific binding of [<sup>3</sup>H]-U69-593 in the presence of 10 $\mu$ M naloxone in the forebrain of WKY and SHR respectively. Note that although [<sup>3</sup>H]-U69-593 had a high non-specific binding to the glass microscope slide, the binding on each brain section was specific for the  $\kappa$ -opioid receptor, as demonstrated by comparison of the specific binding in panels A, B, E-H with the non-specific binding shown in panels C and D. Scale bar represents 2.42mm (A-D), 2.28mm (E, F) or 1.33mm (G, H).



**FIGURE 4.10**

Representative pseudocolour autoradiograms and corresponding brain maps demonstrating the distribution of [<sup>3</sup>H]-U69-593 binding sites at four levels of the rat CNS (A – forebrain: bregma ~ -1.8mm; B – forebrain: bregma ~ -3.1mm; C – pons: bregma ~ -9.7mm; D – medulla oblongata: bregma ~ -13.3mm). For abbreviations, refer to page xvii. Scale bar represents 1.88mm (A and B), 1.45mm (C) and 1.08mm (D).



**FIGURE 4.11**

Comparison of basal (no stress) [ $^3\text{H}$ ]-U69-593 binding site density in the CNS of normotensive (WKY) and hypertensive (SHR) rat strains (n=3 per strain). Density of [ $^3\text{H}$ ]-U69-593 binding sites is expressed as the % change in SHR compared to WKY. For abbreviations, see page xvii.

\*:  $P < 0.05$ ; unpaired student's  $t$ -test.

**% CHANGE IN SHR  
COMPARED TO  
WKY**

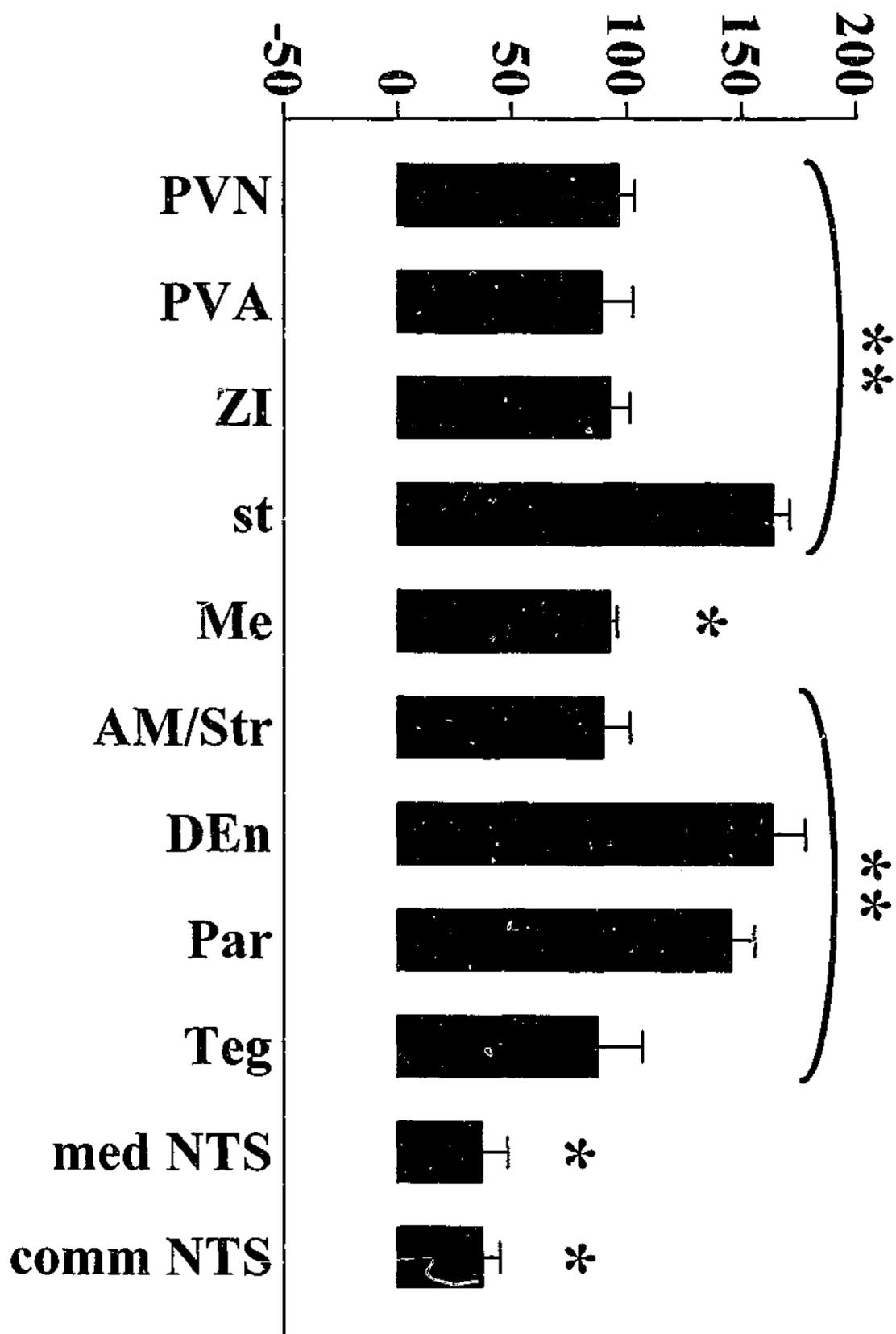


TABLE 4.4

REGION	RELATIVE DENSITY OF [ <sup>3</sup> H]-U69-593 BINDING SITES	
	WKY	SHR
<b>FOREBRAIN</b>		
ARC	++	+++
LHy	++	+++
Pe	++	+++
PVN	++	+++
CM	+	++
PVA	++	+++
ZI	++	+++
st	++	+++
BL	++	+++
Me	++	+++
AM/Str	++	+++
DEn	+	+++
CPu	+	++
Par	+	++
<b>PONS</b>		
Teg	+	++
LC	+	++
SubC	+	++
PnC	+	++
<b>MEDULLA</b>		
med NTS	++	++
comm NTS	++	++
XII	+	+
Sp5C	+	+
IOC	+	+
VLM	+	+

Relative distribution of [<sup>3</sup>H]-U69-593 binding sites in the CNS of WKY and SHR rats. The density of [<sup>3</sup>H]-U69-593 binding sites was assessed as follows: +++ - dense (> 20 DPM/mm<sup>2</sup>); ++ - moderate (10 DPM/mm<sup>2</sup> to 20 DPM/mm<sup>2</sup>) and + - light (< 10 DPM/mm<sup>2</sup>). For abbreviations, see page xvii.

In the pons and medulla,  $\kappa$ -opioid receptor density was quantified in 2 regions – the Tegu in the pons and the NTS in the dorsal medulla oblongata. The Tegu in the SHR strain was found to contain an increased [ $^3\text{H}$ ]-U69-593 binding site density of +87% compared to WKY Tegu ( $P < 0.01$ ; unpaired student's  $t$ -test). As described earlier,  $\kappa$ -opioid receptor density was quantified in the medial and commissural portions of the NTS, and in both of these subregions, SHR rats exhibited significantly elevated  $\kappa$ -opioid receptor density than WKY (+37% in both medial and commissural NTS;  $P < 0.05$ ; unpaired student's  $t$ -test) (Figure 4.11).

#### 4.3.3.2 Restraint stress and [ $^3\text{H}$ ]-U69-593 binding

The effects of acute and chronic restraint stress on  $\kappa$ -opioid receptor density were measured in selected CNS regions in WKY and SHR. As demonstrated in Figure 4.12A, neither acute nor chronic restraint had any effect on [ $^3\text{H}$ ]-U69-593 binding sites in the PVN of WKY. In contrast, 1 and 3 sessions of restraint produced significant decreases of -27% (day 1;  $P < 0.01$ ) and -22% (day 3;  $P < 0.01$ ) in the PVN of SHR. A significant reduction in  $\kappa$ -opioid binding site density was also observed in the PVN of SHR after 5 periods of restraint (-17%;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). While  $\kappa$ -opioid binding was still reduced on the tenth day of the restraint paradigm, this was not significant, suggesting a return towards baseline levels of  $\kappa$ -opioid receptor density. Statistical analysis of the temporal response to restraint in both strains did not reveal any significant interaction between strain and the restraint paradigm ( $F(4,20) = 1.55$ ;  $P > 0.05$ ; two way ANOVA). No significant changes in [ $^3\text{H}$ ]-U69-593 binding site density were observed in the PVA after any period of restraint stress in either strain (Figure 4.12B).

In the Me, acute and chronic restraint elicited decreases in  $\kappa$ -opioid receptor density in both strains (Figure 4.12C). While these decreases were not significant in WKY, possibly due to the large variation in density in the control group, a sustained significant reduction of ~ -30% was observed in the Me of SHR throughout the restraint paradigm ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test). Due to the close similarity in temporal response profile in both strains, a significant interaction between strain and number of sessions of restraint was not found ( $F(4,20) = 0.52$ ;  $P > 0.05$ ; two way ANOVA).

**FIGURE 4.12**

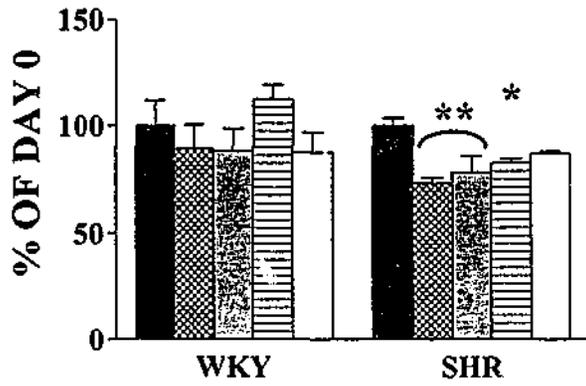
The effect of acute and chronic restraint stress on the density of [<sup>3</sup>H]-U69-593 binding sites in selected regions of normotensive (WKY) and hypertensive (SHR) rat CNS. Rats were exposed to a 60 min period of restraint stress for 0 (control; filled black columns), 1 (hatched columns), 3 (filled grey columns), 5 (horizontal lined columns) or 10 (open columns) consecutive days (n=3 rats per group per strain). Brain regions represented are: A – PVN; B – PVA; C – Me; D – Par; E – med NTS and F – comm NTS. Results are expressed as a percentage of day 0 (control) levels ± S.E.M. For abbreviations, refer to page xvii.

\*:  $P < 0.05$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

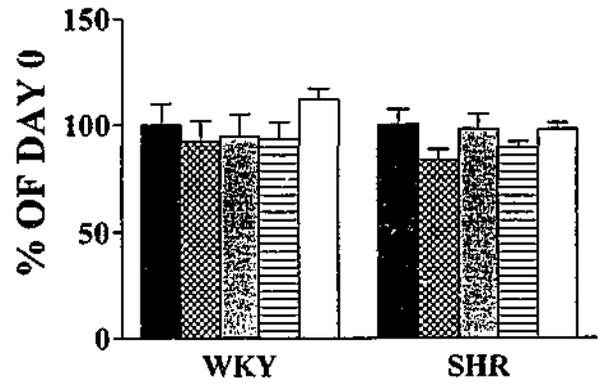
\*\* :  $P < 0.01$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

†:  $P < 0.05$  when comparing the temporal response to restraint in SHR with WKY; two way ANOVA.

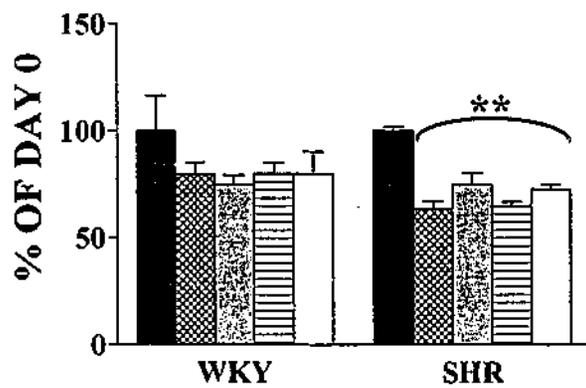
### A - PVN



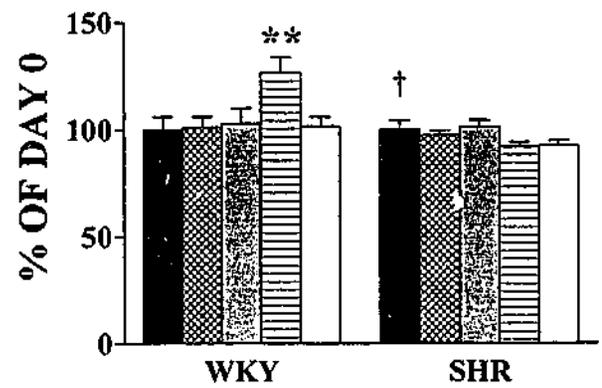
### B - PVA



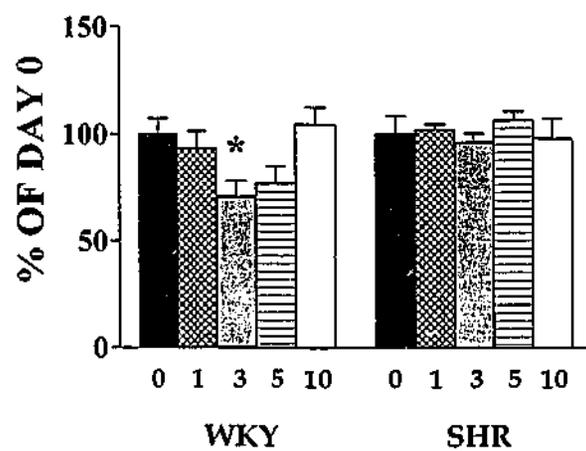
### C - Me



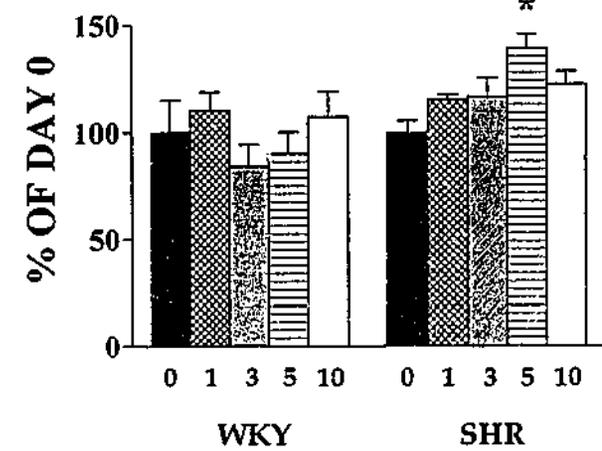
### D - Par



### E - med NTS



### F - comm NTS



A significantly different temporal response to restraint, however, was observed in the Par ( $F(4,20) = 5.06$ ;  $P < 0.05$ ; two way ANOVA). A significant increase of +27% was detected in the Par of WKY after 5 days of the restraint paradigm, and  $\kappa$ -opioid receptor density in the WKY Par did not differ from baseline levels on any other day of the restraint paradigm ( $P < 0.01$ ; one way ANOVA with post-hoc Dunnett's test). In SHR, acute and chronic restraint did not produce any changes in [ $^3\text{H}$ ]-U69-593 binding site density in the Par (Figure 4.12D).

As shown in Figure 4.12E and 4.12F, the restraint paradigm produced an interesting response in the NTS of both strains. In the medial NTS of WKY, the 10 day restraint protocol produced a bell-shaped temporal response profile, with a significant decrease in  $\kappa$ -opioid receptor density observed only after 3 periods of restraint. No changes were detected at any time during 10 days of restraint stress in the medial NTS of SHR. However in the commissural NTS of SHR, 1 and 3 periods of restraint elicited non-significant elevations in  $\kappa$ -opioid receptor density that was followed by a significant increase of +39% on the fifth day of the restraint paradigm ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). After 10 periods of restraint, the density of  $\kappa$ -opioid receptors in the SHR commissural NTS appeared to be returning to no-stress levels. In the WKY commissural NTS, neither acute nor repeated restraint had a significant effect on  $\kappa$ -opioid receptor density.

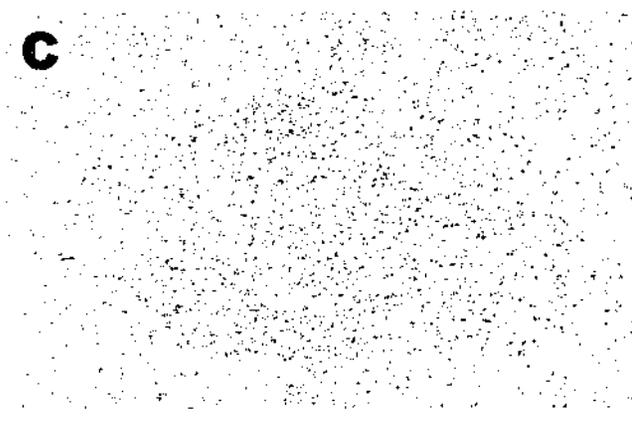
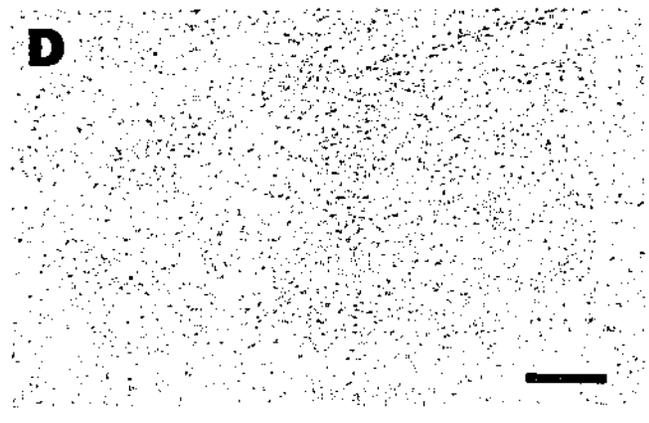
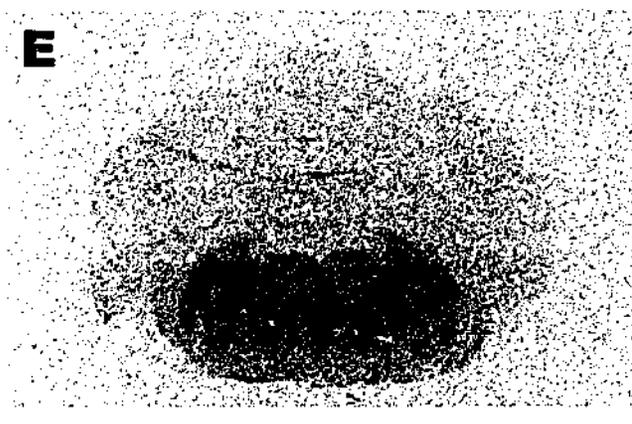
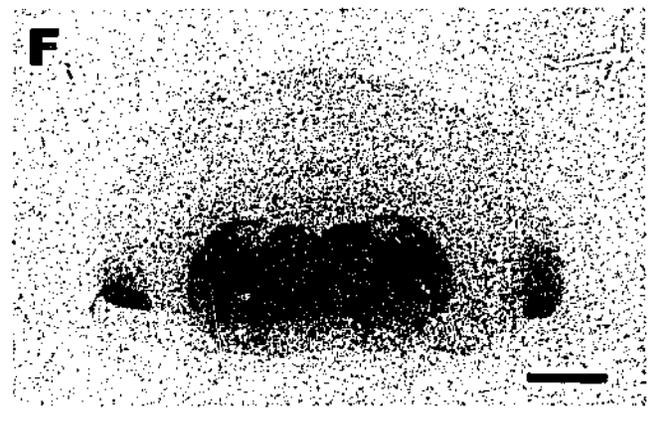
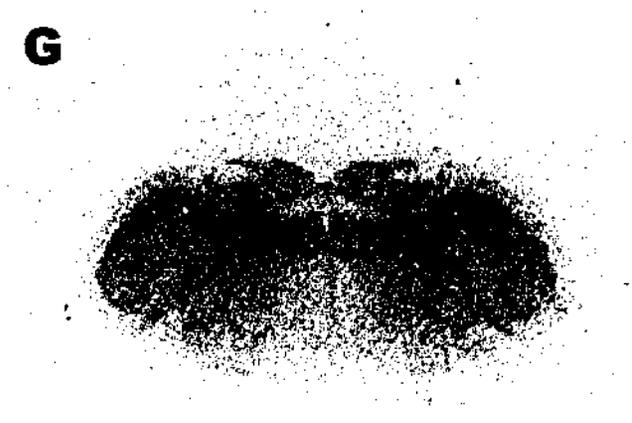
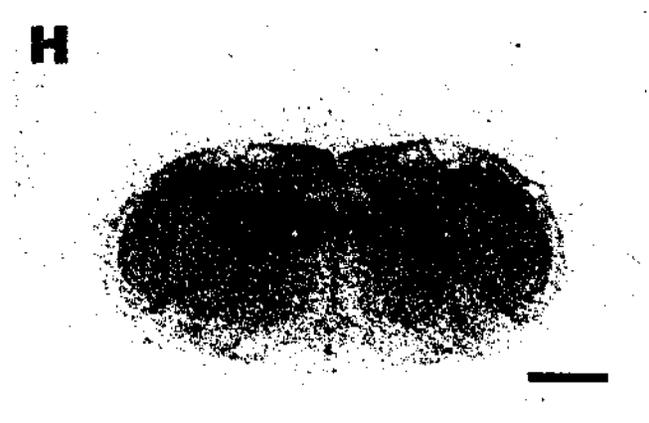
### 4.3.4 GAL RECEPTOR AUTORADIOGRAPHY

#### 4.3.4.1 Strain comparison

Binding sites for [ $^{125}\text{I}$ ]-GAL were detected in numerous nuclei throughout the CNS of both normotensive (WKY) and hypertensive (SHR) rats (Figure 4.13). In the forebrain, [ $^{125}\text{I}$ ]-GAL binding sites were found in the amygdala (Me, Ce, BL and BM), VMH, Pir, st, thalamus (centrolateral (CL) and centromedial (CM)), reuniens nucleus (Re) and ZI (Figure 4.14A and Table 4.5). Pontine nuclei that contained [ $^{125}\text{I}$ ]-GAL binding sites included the PB, Teg, raphe pallidus, RMag and the motor trigeminal nucleus. [ $^{125}\text{I}$ ]-GAL binding was also found in medullary nuclei such as the dorsolateral NTS, Sp5C, XII and reticular fields (Figure 4.14D and Table 4.6). The distribution of [ $^{125}\text{I}$ ]-GAL binding was similar throughout the CNS of both strains (Figure 4.13). As shown in Figure 4.15 and 4.16, statistical comparison of the basal levels of [ $^{125}\text{I}$ ]-GAL binding sites between strains revealed significant decreases in the amygdala

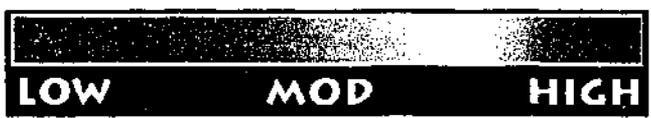
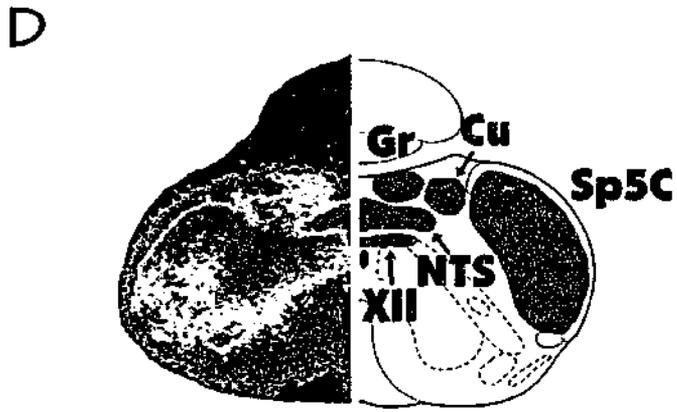
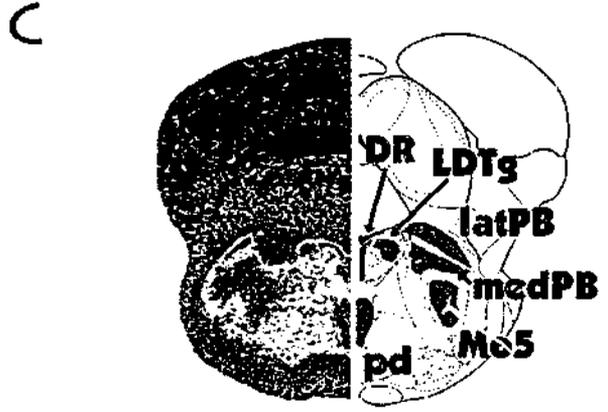
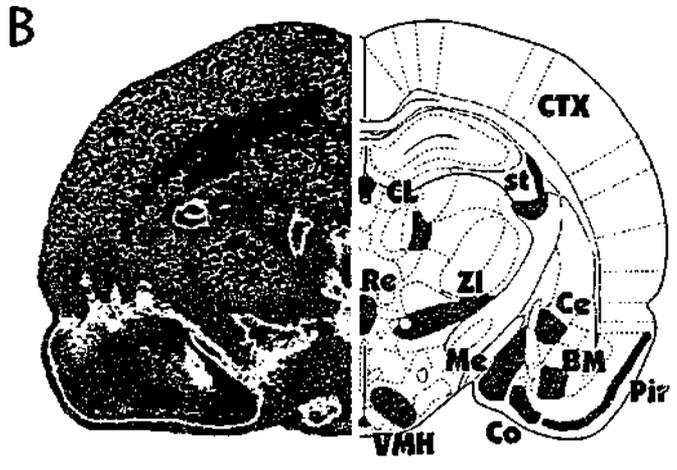
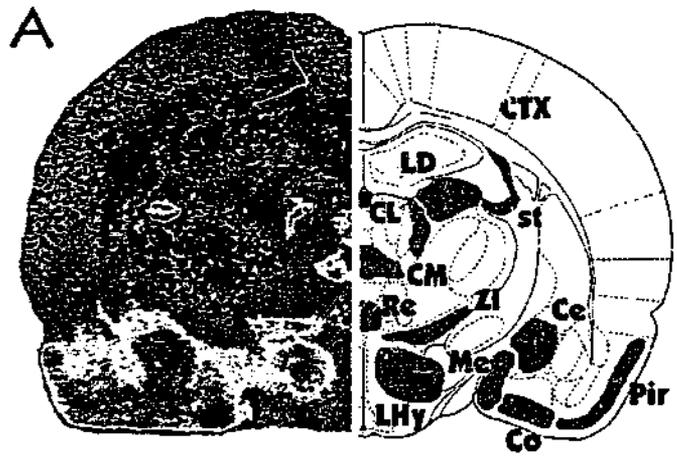
**FIGURE 4.13**

Autoradiographic images of [ $^{125}$ I]-GAL binding site distribution in the forebrain (A-D), pons (E, F) and medulla oblongata (G, H) of normotensive (WKY; A, C, E, G) and hypertensive (SHR; B, D, F, H) rat brain. Panels C and D represent the non-specific binding of [ $^{125}$ I]-GAL in the presence of unlabeled rat GAL in the forebrain of WKY and SHR respectively. Scale bar represents 2.42mm (A-D), 2.28mm (E, F) or 1.33mm (G, H).

**A****B****C****D****E****F****G****H**

**FIGURE 4.14**

Representative pseudocolour autoradiograms and corresponding brain maps demonstrating the distribution of [<sup>125</sup>I]-GAL binding sites at four levels of the rat CNS (A – forebrain: bregma ~ -2.3mm; B – forebrain: bregma ~ -3.1mm; C – pons: bregma ~ -9.2mm; D – medulla oblongata: bregma ~ -14.1mm). For abbreviations, refer to page xvii. Scale bar represents 1.88mm (A and B), 1.45mm (C) and 1.08mm (D).

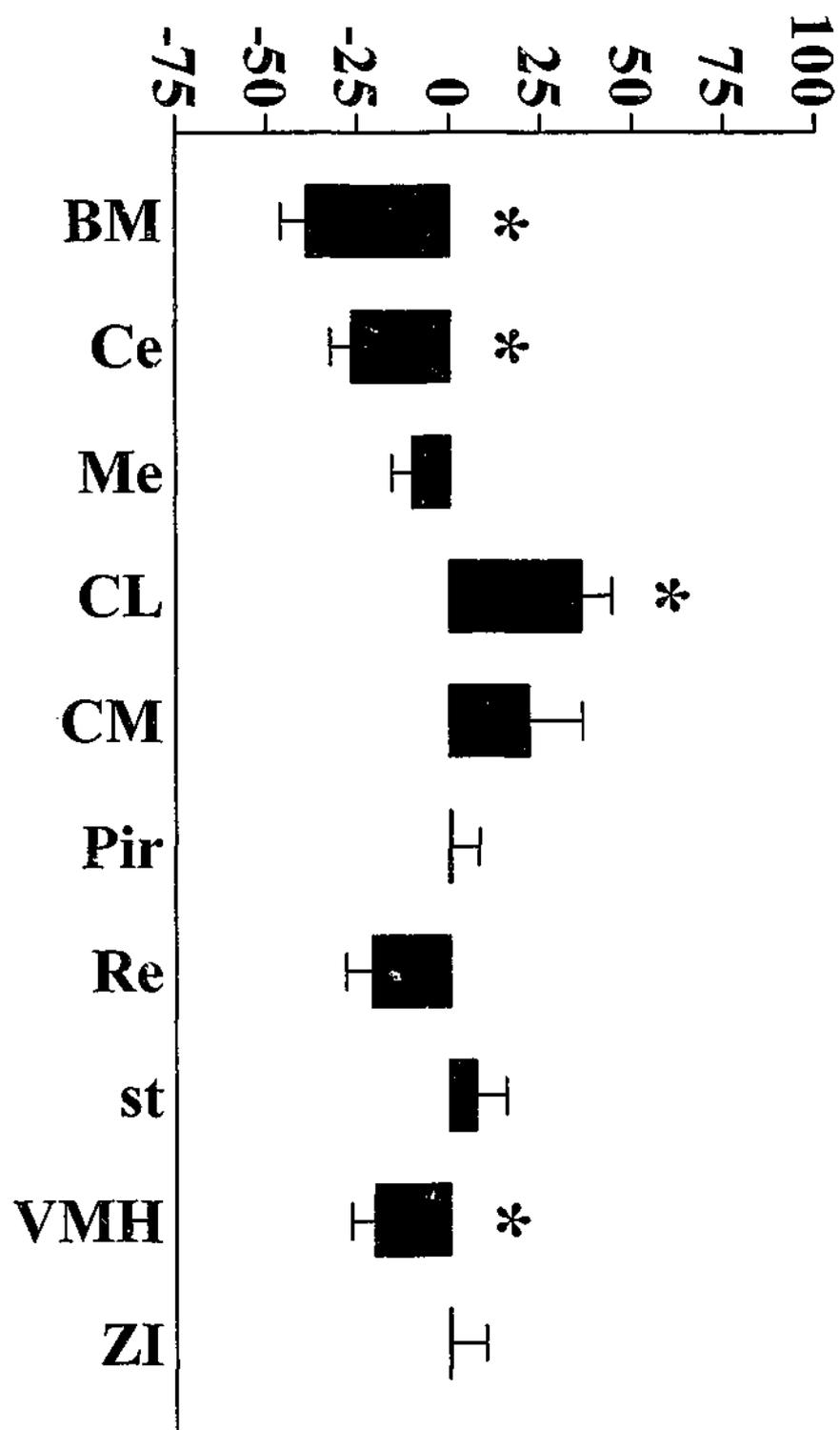


**FIGURE 4.15**

Comparison of basal (no stress) [ $^{125}$ I]-GAL binding site density in the forebrain of normotensive (WKY) and hypertensive (SHR) rat strains (n=3 per strain). Density of [ $^{125}$ I]-GAL binding sites is expressed as the % change in SHR compared to WKY. For abbreviations, see page xvii.

\*:  $P < 0.05$ ; unpaired student's *t*-test.

**% CHANGE IN SHR  
COMPARED TO  
WKY**

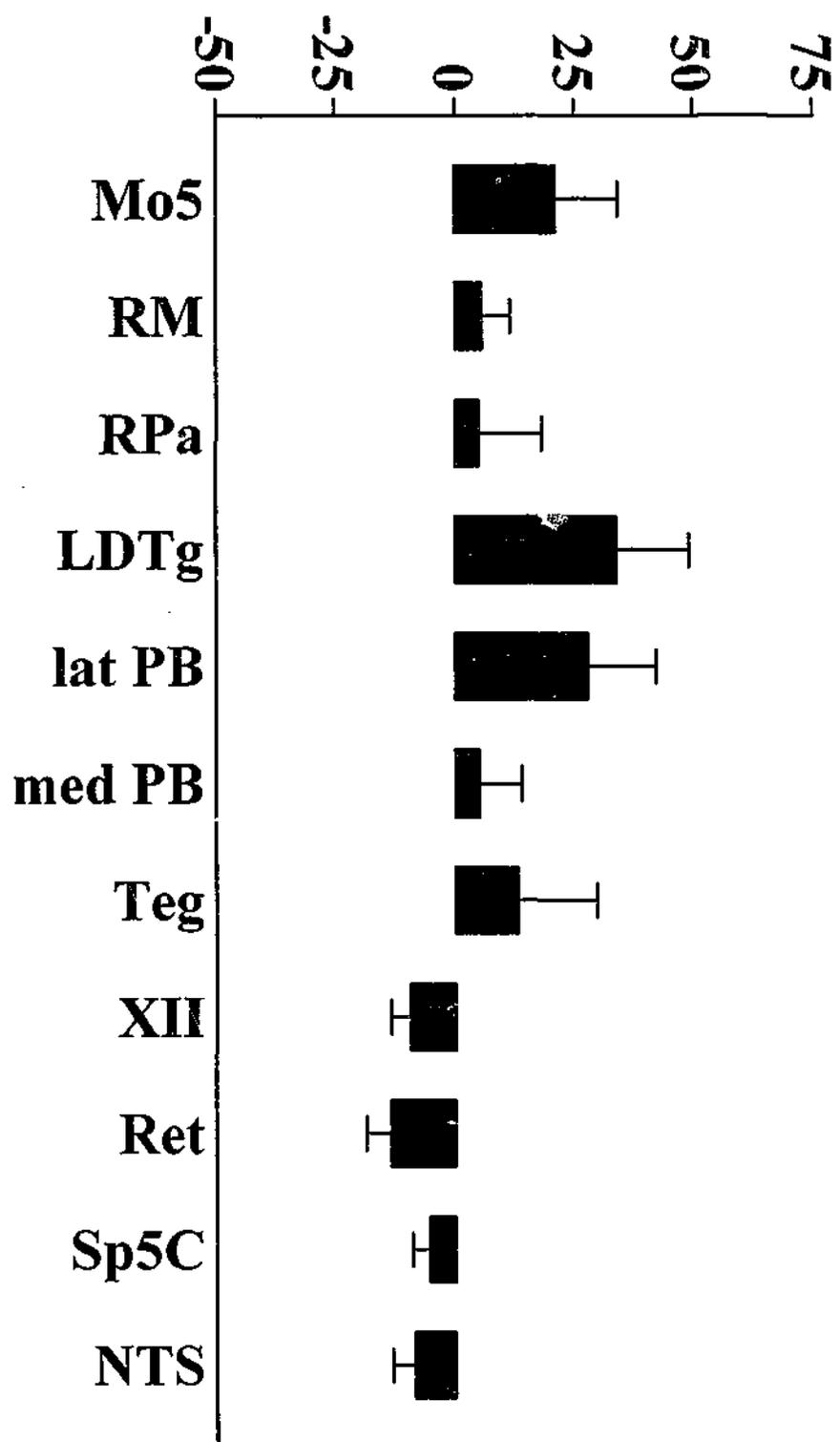


**FIGURE 4.16**

Comparison of basal (no stress) [ $^{125}$ I]-GAL binding site density in the pons/medulla oblongata of normotensive (WKY) and hypertensive (SHR) rat strains (n=3 per strain). Density of [ $^{125}$ I]-GAL binding sites is expressed as the % change in SHR compared to WKY. For abbreviations, see page xvii.

\*:  $P < 0.05$ ; unpaired student's *t*-test.

**% CHANGE IN SHR  
COMPARED TO  
WKY**



(Ce [-27%;  $P < 0.05$ ] and BM [-39%;  $P < 0.01$ ]) and VMH (-20%;  $P < 0.05$ ) in the hypertensive SHR (student's unpaired  $t$ -test). In addition, a significant increase (+36%;  $P < 0.01$ ; student's unpaired  $t$ -test) in [ $^{125}$ I]-GAL binding in the CL was observed in SHR when compared to WKY rats.

TABLE 4.5

REGION	RELATIVE DENSITY OF [ $^{125}$ I]-GAL BINDING SITES	
	WKY	SHR
FOREBRAIN		
BM	+++	+++
Ce	+++	+++
Co	+++	+++
Me	+++	+++
CL	+	++
CM	+	++
LD	+	++
Re	+	+
ZI	++	++
st	++	++
LHy	++	+++
VMH	++	+
Fr	+	+
Pir	+++	+++

Relative distribution of [ $^{125}$ I]-GAL binding sites in the forebrain of WKY and SHR rats. The density of [ $^{125}$ I]-GAL binding sites was assessed as follows: +++ - dense ( $> 45$  DPM/mm $^2$ ); ++ - moderate (30 DPM/mm $^2$  to 45 DPM/mm $^2$ ) and + - light ( $< 30$  DPM/mm $^2$ ). For abbreviations, see page xvii.

TABLE 4.6

REGION	RELATIVE DENSITY OF [ <sup>125</sup> I]-GAL BINDING SITES	
	WKY	SHR
<b>PONS</b>		
Mo5	+	+
DR	+	+
RMag	+	++
RPa	+	+
LDTg	+	+
pd	+	+
Lat PB	+	++
Med PB	+	++
Teg	+	+
<b>MEDULLA</b>		
XII	+++	+++
Rt	+++	++
Sp5C	+++	+++
Gr	++	++
Cu	++	++
NTS	++	++

Relative distribution of [<sup>125</sup>I]-GAL binding sites in the pons and medulla oblongata of WKY and SHR rats. The density of [<sup>125</sup>I]-GAL binding sites was assessed as follows: +++ - dense (> 45 DPM/mm<sup>2</sup>); ++ - moderate (30 DPM/mm<sup>2</sup> to 45 DPM/mm<sup>2</sup>) and + - light (< 30 DPM/mm<sup>2</sup>). For abbreviations, see page xvii.

#### 4.3.4.2 Restraint stress and [<sup>125</sup>I]-GAL binding

Restraint stress elicited changes in [<sup>125</sup>I]-GAL binding site density in numerous CNS nuclei in both strains. Compared to unstressed controls, WKY rats exposed to 1 period of restraint exhibited significant decreases in the amygdala in the BM (-40%), Ce (-41%) and Me (-41%) (Figure 4.17A and B;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). In the Ce of

**FIGURE 4.17**

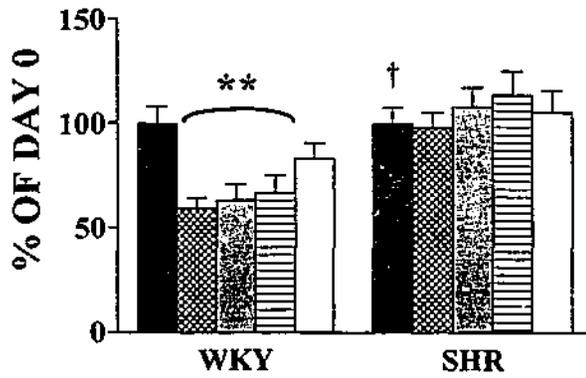
The effect of acute and chronic restraint stress on the density of [<sup>125</sup>I]-GAL binding sites in selected regions of normotensive (WKY) and hypertensive (SHR) rat CNS. Rats were exposed to a 60 min period of restraint stress for 0 (control; filled black columns), 1 (hatched columns), 3 (filled grey columns), 5 (horizontal lined columns) or 10 (open columns) consecutive days (n=3 rats per group per strain). Brain regions represented are: A – Ce; B – Me; C – VMH; D – Pir; E – lat PB; F – NTS; G – Sp5C and H – XII. Results are expressed as a percentage of day 0 (control) levels ± S.E.M. For abbreviations, refer to page xvii.

\*:  $P < 0.05$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

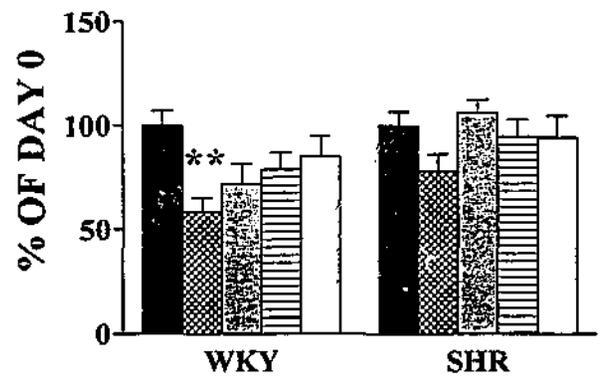
\*\* :  $P < 0.01$  when compared with day 0 (control) levels; one way ANOVA with post hoc Dunnett's test.

†:  $P < 0.05$  when comparing the temporal response to restraint in SHR with WKY; two way ANOVA.

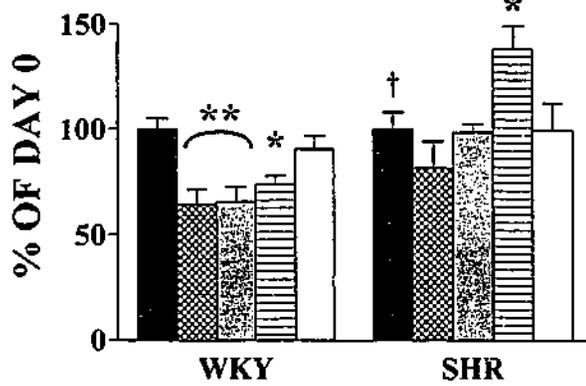
### A - Ce



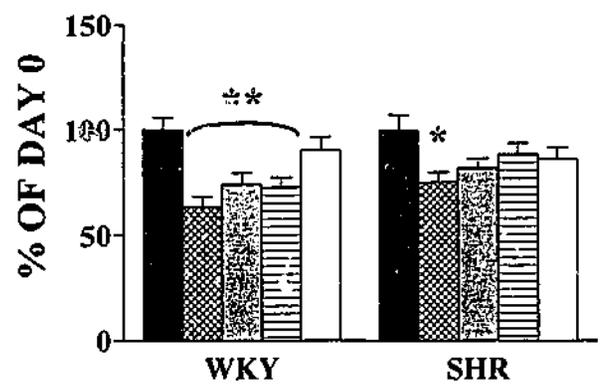
### B - Me



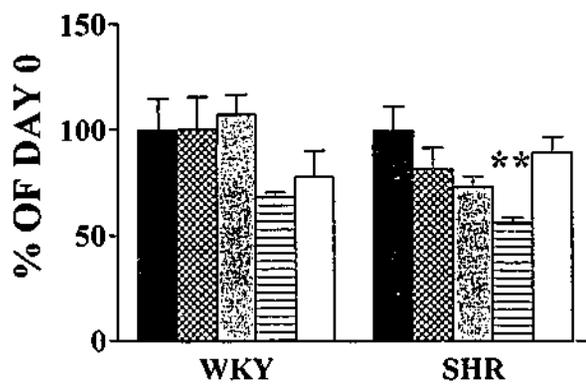
### C - VMH



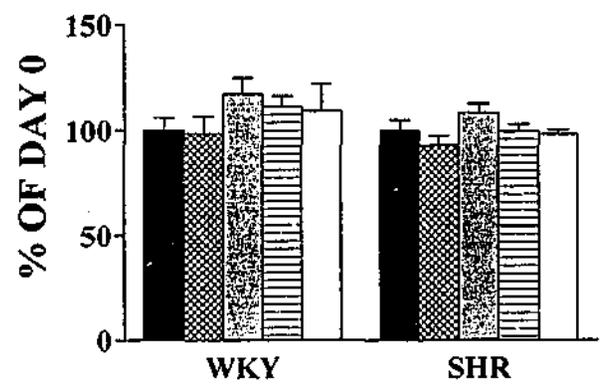
### D - Pir



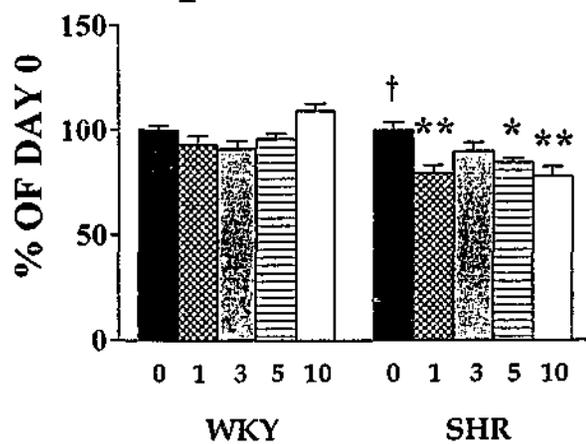
### E - lat PB



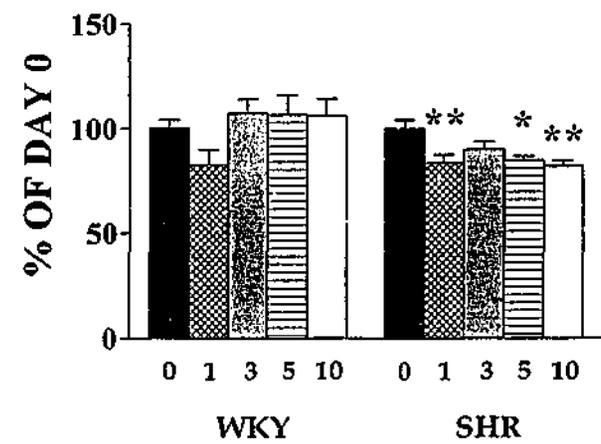
### F - NTS



### G - Sp5C



### H - XII



WKY, continued exposure to the restraint paradigm produced a significant reduction in the density of [ $^{125}$ I]-GAL binding sites (-33%; 5 sessions;  $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). On the tenth day of the restraint paradigm, the decreases in binding sites for [ $^{125}$ I]-GAL in these amygdaloid nuclei (BM, Ce, Me) in WKY are not significant, and consequently the density of [ $^{125}$ I]-GAL receptors is comparable to basal (no stress) levels. In the amygdala of SHR, no changes in [ $^{125}$ I]-GAL binding site density were detected at any time during the restraint paradigm. Statistical comparison between WKY and SHR revealed the presence of a significant interaction between strain and the number of restraint sessions in the Ce and BM (BM -  $F(4,20) = 2.58$  and Ce -  $F(4,20) = 2.64$ ; both  $P < 0.05$ ; two way ANOVA).

In the VMH, significant restraint-induced changes in GAL receptor density were evident in both strains (Figure 4.17C). In WKY, acute (1 session) and chronic (5 sessions) restraint produced significant reductions in the density of [ $^{125}$ I]-GAL binding sites of -35% and -26% respectively ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). In contrast, 5 periods of restraint produced a significant increase (+39%) in [ $^{125}$ I]-GAL binding site density in the VMH of SHR ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). Moreover, the temporal response of the VMH to restraint was significantly different between WKY and SHR ( $F(4,20) = 4.42$ ;  $P < 0.01$ ; two way ANOVA).

In the pons and medulla, significant changes in [ $^{125}$ I]-GAL receptor binding were more widespread in SHR than WKY. Five periods of restraint induced a significant decrease in the lateral PB (-43%) of SHR (Figure 4.17E). In WKY, while no significant changes were observed in the lateral subregion of PB, a significant reduction in [ $^{125}$ I]-GAL binding sites was detected in the medial PB (-29%) after 10 sessions of restraint ( $P < 0.05$ ; one way ANOVA with post-hoc Dunnett's test). No further changes in the density of [ $^{125}$ I]-GAL binding sites were detected in the medulla of WKY, with regions such as the NTS maintaining a constant level of [ $^{125}$ I]-GAL binding throughout the restraint paradigm (Figure 4.17F). In contrast, significant decreases in [ $^{125}$ I]-GAL binding were observed in the XII and Sp5C of SHR over the entire 10 day restraint period (-15% to -18% [XII] and -15% to -22% [Sp5C]) (Figure 4.16G and 4.16H). However, in both nuclei in the SHR, 3 periods of restraint induced a non-significant decrease in [ $^{125}$ I]-GAL binding, suggesting that a biphasic response to restraint may exist. In addition, a comparison of the temporal response to the restraint paradigm revealed that a significant interaction between strain and the number of restraint sessions existed in Sp5C ( $F(4,20) = 6.16$ ;  $P < 0.001$ ), but not in the XII ( $F(4,20) = 2.39$ ;  $P > 0.05$ ; two way ANOVA).

## 4.4 DISCUSSION

### 4.4.1 AUTORADIOGRAPHY PROTOCOL CONSIDERATIONS

Before commencing each of the autoradiography experiments detailed above, preliminary wipe tests were completed to optimise experimental conditions such that the specific binding of each ligand was maximal. A single concentration of each radioligand that targetted the three opioid receptor subtypes ( $\mu$ ,  $\delta$  and  $\kappa$ ) and GAL receptors was then selected on this basis and used in the subsequent autoradiography studies comparing the density of opioid and GAL receptors in the CNS of WKY and SHR in the basal and stressed state. Furthermore, the concentration of radioligand used in each experiment was comparable to the reported  $K_d$  value in rat brain (McLean *et al.*, 1986; Skofitsch *et al.*, 1986; Nock *et al.*, 1990; Yamamura *et al.*, 1992). Using this approach, a specific and distinct distribution was observed for each of the 4 radioligands in the rat CNS. Furthermore, the 10 day restraint paradigm produced strain-, region- and ligand-specific temporal response profiles in the CNS of WKY and SHR. Significant restraint-induced changes in ligand binding ranged from approximately 10% up to 50%, and may represent changes in receptor affinity, density or both. However, the magnitude of the alterations in ligand binding induced by restraint suggest that these can be attributed mainly to changes in receptor density, although contributions from receptor affinity alterations cannot be ruled out.

As only one concentration for each ligand was used, there is room for further research into the neurochemistry of each of the receptor systems in the CNS of WKY and SHR. Binding experiments using membrane preparations or slide-mounted sections over a range of ligand concentrations could be utilised to provide accurate measurements of  $B_{MAX}$  and  $K_d$  for the  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid and GAL receptor ligands in WKY and SHR. In addition, this study could be extended to measure  $B_{MAX}$  and  $K_d$  values in specific brain regions of WKY and SHR rats that have been exposed to a restraint paradigm similar to the one used in the present thesis. The results from studies such as these would therefore provide more information regarding stress-induced changes in opioid and GAL receptor affinity and density in WKY and SHR.

## 4.4.2 $\mu$ -OPIOID RECEPTOR AUTORADIOGRAPHY

### 4.4.2.1 Strain comparison

The iodinated ligand [ $^{125}$ I]-FK 33-824 was used to label  $\mu$ -opioid receptors throughout the CNS of both normotensive and hypertensive rats. In both strains, a similar distribution profile was observed. Previous autoradiographic studies using [ $^{125}$ I]-FK 33-824 have focussed on the  $\mu$ -opioid receptor populations in the forebrain (Rothman *et al.*, 1987; Cowen *et al.*, 1999). Cowen and co-workers (Cowen *et al.*, 1999) and the present study visualised [ $^{125}$ I]-FK 33-824 binding sites in similar forebrain regions, including the amygdala, cortex and thalamus, although the study by Cowen and colleagues provides a much more detailed quantification of the distribution of  $\mu$ -opioid receptors within these regions (Cowen *et al.*, 1999). In addition, investigations of the central distribution of the  $\mu$ -opioid receptor have also been reported using other ligands such as [ $^3$ H]-DAGO, [ $^3$ H]-CTOP and [ $^3$ H]-ohmefentanyl, and there is a good correspondence between these reports and the present study despite the use of different radioligands (Hawkins *et al.*, 1988; Mansour *et al.*, 1994c; Yin *et al.*, 1996a).

Although this study is the first report of pontine and medullary [ $^{125}$ I]-FK 33-824 binding sites, there have been previously published studies detailing the distribution of  $\mu$ -opioid receptors in these CNS regions using alternative ligands (Mansour *et al.*, 1994c; Yin *et al.*, 1996a). Once more, there is a correlation between the distribution of  $\mu$ -opioid receptor populations in regions such as the PB, Namb, NTS, Gr and Cu. In contrast to the study by Mansour and colleagues (Mansour *et al.*, 1994c) where  $\mu$ -opioid receptors were not visualised in the cerebellum, a low density of [ $^{125}$ I]-FK 33-824 binding sites were detected in the cerebellum in the present study.

Despite similarities in the central distribution of  $\mu$ -opioid receptors between strains, significantly altered levels of  $\mu$ -opioid receptors were detected in a number of nuclei of SHR compared to WKY, including the BL, thalamus, Hab, cortex and cerebellum. In the BL of the amygdala, a significantly increased density of [ $^{125}$ I]-FK 33-824 binding sites was observed in SHR compared to WKY. This result is in contrast to the findings of Gulati and co-workers (Gulati & Bhargava, 1990) who reported a reduced content of  $\mu$ -opioid receptors in the grossly dissected amygdala, although the density of receptors in the BL alone cannot be inferred from that study. Furthermore, a significantly decreased level of  $\mu$ -opioid receptors were detected in

the BL of SHR compared to WKY (Yin *et al.*, 1996a). Reasons that may account for the differences in results between the present study and Yin and colleagues (Yin *et al.*, 1996a) may be related to the ligand used, although both appear to be selective for the  $\mu$ -opioid receptor. Since both ligands are agonists at the  $\mu$ -opioid receptor, it is feasible that each ligand may be recognising distinct conformational states of the receptor. In support, one study has demonstrated that [ $^{125}$ I]-FK 33-824 binds to two separate affinity states of the  $\mu$ -opioid receptor,  $\beta$ -funaltrexamine-sensitive and -insensitive sites (Rothman *et al.*, 1987). Thus, [ $^3$ H]-ohmefentanyl which was used in the study by Yin and co-workers (Yin *et al.*, 1996a), may recognise only one of these states of  $\mu$ -opioid receptors.

In the forebrain at the level of the PVN, the thalamus contained the largest population of  $\mu$ -opioid receptors. Furthermore, when the density of these thalamic  $\mu$ -opioid receptors were compared between normotensive and hypertensive rats, the present study revealed that SHR contained a significantly lower density when compared to WKY. The Hab also contained a significantly lower number of [ $^{125}$ I]-FK 33-824 binding sites in SHR compared to WKY. Previous studies preferred to focus on thalamic subnuclei rather than the entire structure, with Yin and colleagues (Yin *et al.*, 1996a) also reporting that the Hab of SHR contained a lower density of  $\mu$ -opioid receptors than WKY. A study completed by Kujirai and colleagues (Kujirai *et al.*, 1991) reported a slightly contrasting result, with an elevated density of  $\mu$ -opioid receptors located in the lateral posterior thalamic nucleus of SHR compared to WKY. As the present investigation did not delineate thalamic subnuclei, it is possible that  $\mu$ -opioid receptor density may also be elevated in the lateral posterior thalamic nucleus of SHR. In addition, Kujirai and co-workers compared the central  $\mu$ -opioid receptor density of SHR with the normotensive SD strain, rather than the WKY strain used in the present study, and this methodological difference may account for discrepancies in the data between the two studies (Kujirai *et al.*, 1991).

Inputs to the thalamus arise from regions such as the amygdala, including the BL and Ce, ARC, PAG, dorsal raphe, LC, PB, NTS, VLM and sp5 (Sim & Joseph, 1991; Lechner *et al.*, 1993; Williams *et al.*, 1994; Otake & Nakamura, 1995; Reardon & Mitrofanis, 2000). Efferent projections of the thalamus include the NAcc, CPu, various amygdaloid nuclei and frontal motor cortex (Berendse & Groenewegen, 1990; Turner & Herkenham, 1991; Deschenes *et al.*, 1996). Thus, the extensive afferent and efferent projections of the thalamus suggest that alterations in neuronal activity produced by  $\mu$ -opioid receptor density changes may have effects throughout the CNS of SHR.

The cortex was sampled as a single structure and consisted of the RS, Fr, Par and Pir. In SHR, these cortical subregions were found to contain a significantly lower density of  $\mu$ -opioid receptors when compared to WKY, a result that is supported in part by a study completed by Yin and co-workers (Yin *et al.*, 1996a), where a reduced density of  $\mu$ -opioid receptors were detected in the posterior Cing of SHR when compared to WKY. In contrast, Kujirai and colleagues (Kujirai *et al.*, 1991) reported that the anterior Cing of SHR contained an elevated  $\mu$ -opioid receptor density when compared to WKY. The present study did not attempt to quantify [ $^{125}$ I]-FK 33-824 binding sites in the Cing, so it is unknown whether the density of  $\mu$ -opioid receptors would be significantly increased or decreased in this region in the SHR strain compared to the normotensive controls. Furthermore, a study completed by Gulati and co-workers did not detect any differences in the density of  $\mu$ -opioid receptors in cortical membranes between WKY and SHR using [ $^3$ H]-DAGO as the  $\mu$ -opioid selective ligand (Gulati & Bhargava, 1990).

In SHR, the rostral cerebellum was found to contain a significantly elevated level of  $\mu$ -opioid receptor binding compared to their normotensive counterparts. The present study represents the first report of alterations in cerebellar  $\mu$ -opioid receptor density, as previous comparisons did not quantify  $\mu$ -opioid receptor binding in this region (Gulati & Bhargava, 1990; Kujirai *et al.*, 1991; Yin *et al.*, 1996a). This was surprising given that  $\mu$ -opioid receptor mRNA and endomorphin-2-ir have previously been detected in the cerebellum (Brodsky *et al.*, 1995; Pierce & Wessendorf, 2000). Moreover, functional data demonstrated that intra-cerebellar administration of morphine, a  $\mu$ -opioid receptor agonist, produced analgesia (Dey & Ray, 1982). Thus, elevated  $\mu$ -opioid receptor density in the cerebellum may contribute to the hypoalgesic state previously observed in SHR (Sitsen & de Jong, 1983).

Strain-related alterations in the density of  $\mu$ -opioid receptors were limited to the BL, Hab, thalamus, cortex and cerebellum in the present study. According to previously published data, SHR also contain elevated densities of the  $\mu$ -opioid receptor in regions such as the CPu, VTA, DG and PAG compared to normotensive rats (Kujirai *et al.*, 1991).  $\mu$ -Opioid receptor density was not measured in these regions in the present study, and further experiments using [ $^{125}$ I]-FK 33-824 and additional brain sections containing regions such as the rostral CPu, VTA and PAG would therefore compliment the results of the present investigation.

The present study, together with the three previously published comparisons of  $\mu$ -opioid receptor density in the CNS of normotensive and hypertensive rats, have produced varied results

(Gulati & Bhargava, 1990; Kujirai *et al.*, 1991; Yin *et al.*, 1996a). These studies have used [<sup>3</sup>H]-ohmefentanyl, [<sup>3</sup>H]-DAGO and [<sup>125</sup>I]-FK 33-824 as  $\mu$ -selective ligands, suggesting that the ligand chosen to radioactively label central  $\mu$ -opioid receptors and the associated experimental conditions may be important factors influencing the outcome of the strain comparison. A study of the binding characteristics of [<sup>3</sup>H]-ohmefentanyl demonstrated that DAGO was unable to fully displace the binding of [<sup>3</sup>H]-ohmefentanyl (Wang *et al.*, 1991). As haloperidol, a  $\sigma$ -receptor agonist, was able to displace [<sup>3</sup>H]-ohmefentanyl from DAGO-insensitive sites, the authors suggested that [<sup>3</sup>H]-ohmefentanyl may also recognise  $\sigma$ -receptors. Thus, [<sup>3</sup>H]-ohmefentanyl may not be the most selective  $\mu$ -opioid receptor ligand and the discrepant results of Yin and colleagues (Yin *et al.*, 1996a) may be attributed to these additional binding sites. Furthermore, these different ligands may be targetting different states (or subtypes) of the  $\mu$ -opioid receptor, particularly with studies reporting the existence of multiple  $\mu$  receptor subtypes produced by alternative enzymatic processing (splicing) (Abbadie *et al.*, 2000). Further characterisation of the  $\mu$ -opioid receptors and its complexes may therefore provide a better understanding of its molecular biology, distribution and functional roles and assist in an explanation of the varied results reported by these four studies.

#### 4.4.2.2 Effects of restraint stress

Rats from the normotensive and hypertensive strains were exposed to a restraint paradigm that lasted for 1, 3, 5 or 10 consecutive days. This paradigm has been previously shown to induce changes in HR, BP, core body temperature and nociception (Appelbaum & Holtzman, 1986; Chen & Herbert, 1995; McDougall *et al.*, 2000). Restraint also activates neuronal populations throughout the entire neuraxis, together with concurrent changes in neuropeptide precursor mRNA and their receptors (Zeman *et al.*, 1988; Chen & Herbert, 1995; Cullinan *et al.*, 1995; Krukoff *et al.*, 1999). Furthermore, the activity of the central opioid system can be modulated by restraint, with acute and chronic restraint eliciting significant increases and decreases in prepro-ENK and proDYN mRNA, precursors for some of the endogenous opioids (see Chapter 3).

The present study provides further evidence that the central opioid system, in particular  $\mu$ -opioid receptors, can be perturbed by acute and chronic restraint stress. Changes in  $\mu$ -opioid receptor density were observed in the forebrain, pons and medulla of both normotensive and

hypertensive rats following exposure to the restraint stress paradigm. In the amygdala,  $\mu$ -opioid receptor density was quantified in the BL and BM subnuclei. While no changes in the levels of  $\mu$ -opioid receptors were observed in the BM of either strain at any time during the restraint stress paradigm, the BL of SHR was sensitive to the effects of acute and chronic restraint stress. Significant reductions in  $\mu$ -opioid receptor density were observed in the BL of SHR after 1, 3 and 10 periods of restraint, with the largest decrease (-28%) recorded on day 3 and 10. In contrast, no alterations in  $\mu$ -opioid receptor density were detected in the BL of WKY, demonstrating a difference in the temporal response to the restraint paradigm between SHR and WKY.

Stimulation of  $\mu$ -opioid receptors in the rat BL results in hypoalgesia (Helmstetter *et al.*, 1995). The BL, which is either directly or indirectly connected with the PAG and rostral ventromedial medulla (RVMM), appears to be an integral component of the neural circuitry mediating the inhibition of nociceptive reflexes that has often been observed during exposure to stress (Kelly & Franklin, 1987; Stein *et al.*, 1992; Helmstetter *et al.*, 1998). The central opioid system has also been associated with stress-induced analgesia. Analgesia elicited by stressors such as restraint has been attenuated by central administration of selective and non-selective opioid antagonists, while stressors have potentiated the analgesic effects of endogenous opioid peptides (Kelly & Franklin, 1987; Calcagnetti *et al.*, 1990; Calcagnetti *et al.*, 1992). Furthermore, studies have suggested that  $\mu$ -opioid receptors, at the level of the BL or PAG may contribute to the stress-induced analgesia phenomenon (Helmstetter *et al.*, 1995; Helmstetter *et al.*, 1998; Tershner & Helmstetter, 2000).

The present study did not observe any changes in  $\mu$ -opioid receptor density in the amygdala of normotensive rats exposed to stress, supporting the results of an earlier study by Stein and colleagues (Stein *et al.*, 1992). These data therefore suggest that any alterations in the nociceptive system induced by acute and chronic restraint may not be produced by changes in  $\mu$ -opioid receptors at the level of the amygdala. However, the present study found that acute and chronic restraint elicited significant decreases in  $\mu$ -opioid receptors in the BL of SHR. Thus, SHR were found to have an increased density of  $\mu$ -opioid receptors in the BL in the unstressed state, and this pre-existing difference in  $\mu$ -opioid receptor density may contribute to an altered stress response in the BL, with possible associated functional effects on nociception. Widy-Tyszkiewicz and colleagues (Widy-Tyszkiewicz *et al.*, 1995) reported that i.p. administration of naloxone attenuated stress-induced analgesia to a larger extent in SHR than WKY. However,

this type of study, where naloxone was administered peripherally, does not provide an indication of the brain regions involved in the mediation of stress-induced analgesia by the opioid system, if naloxone is indeed acting centrally. The BL is therefore a promising target for further research into stress-induced analgesia and the opioid system in SHR.

The cortical neurons in WKY containing  $\mu$ -opioid receptors appeared to be sensitive to the restraint paradigm, with an initial decrease in [ $^{125}$ I]-FK 33-824 binding site density after 1 period of restraint followed by a return to basal levels on subsequent exposure to restraint. This temporal response pattern appears to share an inverse relationship with *c-fos* mRNA expression in the grossly-dissected cortex of acutely and chronically stressed rats (Melia *et al.*, 1994). In this ISHH study, a significant increase in *c-fos* mRNA levels were observed in the cortex after the first period of restraint, while the expression of the *c-fos* transcript decreased to basal levels following chronic periods of restraint (Melia *et al.*, 1994). The activation of  $\mu$ -opioid receptors often results in inhibition of neuronal activity (Chen *et al.*, 1993a; Brunton & Charpak, 1998). Thus, the reduction in  $\mu$ -opioid receptor density, and an associated decrease in inhibition mediated by the  $\mu$ -opioid receptor, would permit cellular activation during exposure to stress. In addition, the observation that both  $\mu$ -opioid receptor density and *c-fos* mRNA expression returned to resting levels as the number of restraint sessions increased suggests that these neurons, as well as their afferent input, have a functional ability to respond to altered levels of stimulation during the restraint paradigm. In support, studies have demonstrated that Met-enkephalin-ir was reduced in the prefrontal cortex and Pir of rats exposed to acute immobilisation (Kurumaji *et al.*, 1987), while handling, mild restraint and footshock decreased [ $^3$ H]-DAGO binding sites in the Cing and Fr cortices (Stein *et al.*, 1992). However, water deprivation had no significant effect on  $\mu$ -opioid receptors in the Cing and Fr, suggesting that these regions of the cortex may only be involved in mediating neural responses to psychological stimuli that require cognitive processing.

In contrast to the normotensive strain, acute restraint stress had no effect on [ $^{125}$ I]-FK 33-824 binding site density in the cortex of SHR, while chronic restraint elicited a significant reduction in the density of cortical  $\mu$ -opioid receptors. Notably, the basal level of  $\mu$ -opioid receptor density in the cortex of unstressed SHR was similar to the density of  $\mu$ -opioid receptors in the cortex of WKY rats that had been exposed to acute restraint. One consequence of the basal difference in cortical  $\mu$ -opioid receptor density between strains may be a loss of sensitivity, such

that only sustained, chronic exposure to a stressor has the ability to produce significant density changes in cortical  $\mu$ -opioid receptors in SHR.

The various cortical subregions that were present in the grossly sampled cortex in the present study have been associated with a number of physiological processes. The Pir, located in the ventral cortex and lateral to the amygdala was shown to receive neuronal input from the OB (Scott *et al.*, 1980), with lesion of the Pir attenuating olfactory discrimination and associated tasks (Staubli *et al.*, 1987). In addition, the Cing, RS and Pir receive input from the medial prefrontal cortex, BL and RVLm have been shown to provide afferent input to regions of the cortex such as the Cing, Pir and RS (Zagon *et al.*, 1994; Dziewiatkowski *et al.*, 1998).

Anatomical studies have demonstrated that cortical subregions such as the Par project to the medial prefrontal cortex, thalamus, hippocampus and colliculus (Diorio *et al.*, 1993; Shibata, 1994; Shibata, 1998; Garcia Del Cano *et al.*, 2000). The medial prefrontal cortex receives afferent input from nuclei such as the LC and thalamus, with these projections conveying vital information relating to the cardiovascular, motor and emotional state of the rat during stress (Holets *et al.*, 1988; Deschenes *et al.*, 1996; Bubser & Deutch, 1999). Furthermore, the NTS, septum and PB receive neuronal input from the prefrontal cortex, and medial prefrontal cortical afferents course through the medial forebrain bundle which terminate in the PVN at the level of the LHy/PeF (Sawchenko & Swanson, 1983; Van der Kooy *et al.*, 1984; Moga *et al.*, 1990a; Diorio *et al.*, 1993). Thus, any stress-induced changes in  $\mu$ -opioid receptor density in the cortex may have widespread effects throughout the CNS on a number of different functions.

In the PB of SHR, acute and chronic restraint had no effect on  $\mu$ -opioid receptor density. Similarly,  $\mu$ -opioid receptor density was not altered in the PB of WKY after acute restraint, but a significant elevation in [ $^{125}$ I]-FK 33-824 binding site density was detected in the PB of normotensive WKY after 10 sessions of restraint. The observation that  $\mu$ -opioid receptors in the PB of WKY were sensitive only to chronic stress suggests that an increase in the activity of an opioid extrinsic afferent input to the PB, or an intrinsic interneuron, has a functional role in either the chronic stress response or assists in the coping mechanism of WKY. Previous studies of the effects of stress on the PB have reported neuronal activation within the medial and lateral PB following exposure of rats to footshock and peripheral cold, heat and pain stimuli (Cullinan *et al.*, 1995; Menendez *et al.*, 1996). The PB appears to act as a relay between the spinal cord and higher brain centres, with direct enkephalin- and dynorphin-containing projections from the spinal cord terminating in the PB (Standaert *et al.*, 1986). Furthermore, systemic administration

of morphine suppressed the activity of PB neurons stimulated by peripheral noxious stimuli (Menendez *et al.*, 1996). While this observation suggests that  $\mu$ -opioid receptors mediate neuronal inhibition, a finding also reported by previous studies (Christie & North, 1988), it is not clear whether these  $\mu$ -opioid receptors are located within the PB or in another central region that has a modulatory influence on PB neurons. A study where  $\mu$ -opioid receptor agonists and antagonists are microinjected into the PB would provide a clearer understanding of the association between  $\mu$ -opioid receptors and functions associated with the PB, including nociception and respiration (Chamberlin & Saper, 1994; Menendez *et al.*, 1996). In the PB,  $\mu$ -opioid receptors have been detected on neurons that project to the Ce, while enkephalin-containing terminals in the PB have been visualised in close proximity to neurons projecting to the ARC, thus providing evidence that changes in  $\mu$ -opioid receptor density may alter the activity of ARC and Ce neurons and subsequent functions mediated by these regions (Magoul *et al.*, 1993; Chamberlin *et al.*, 1999).

In the present study, acute and chronic restraint stress had no significant effect on  $\mu$ -opioid receptor density in the medial NTS of either WKY or SHR rats. In contrast, the commissural NTS of WKY exhibited a significant decrease in the density of [ $^{125}$ I]-FK 33-824 binding sites after 5 and 10 sessions of restraint. Moreover, this response was not observed in the hypertensive strain, with neither acute nor chronic restraint stress producing any changes in  $\mu$ -opioid receptor density in the commissural NTS of SHR. The medial NTS, which consists of the rostral and part of the subpostremal level of the NTS, receives projections from a wide range of visceral afferents, including baroreceptors, chemoreceptors, pulmonary receptors and the various receptors of the gastrointestinal tract (see Barraco *et al.*, 1992). While the commissural NTS does not receive such a dense innervation from visceral afferents, it does receive projections from baroreceptors, chemoreceptors and pulmonary receptors (Barraco *et al.*, 1992).  $\mu$ -Opioid receptors have been localised on vagal afferent axon terminals in the NTS, suggesting that  $\mu$ -opioid receptors are in a position to regulate vagal input to the NTS (Aicher *et al.*, 2000). Electrophysiological studies using  $\mu$ -selective agonists have demonstrated that stimulation of  $\mu$ -opioid receptors within the NTS can modulate vagal neurotransmission, with the primary effect being inhibition and an associated increase in  $K^+$  conductance (Rhim *et al.*, 1993). Functionally, microinjection of  $\mu$ -agonists such as DAGO or FK 33-824 into the NTS of anaesthetised rats depresses respiratory rate, causes hypotension and bradycardia and attenuates the baroreflex elicited by stimulation of the aortic depressor nerve (Gordon, 1990; Rabkin, 1991).

Consequently, restraint-induced decreases in  $\mu$ -opioid receptors in the commissural NTS may result in concomitant alterations in the cardiovascular and respiratory systems.

In SHR, there may be a shift in the properties of  $\mu$ -opioid receptors in the NTS, with one study demonstrating that the hypotension and bradycardia elicited by intra-NTS  $\beta$ -endorphin were blocked by a  $\delta$ -opioid antagonist (ICI 174864) in SHR, and not by a  $\mu$ -selective antagonist ( $\beta$ -funaltrexamine) (Mosqueda-Garcia & Kunos, 1987). In contrast, the cardiovascular effects of intra-NTS administration of  $\beta$ -endorphin in normotensive SD rats were inhibited by  $\beta$ -funaltrexamine, while ICI 174864 had no effect (Mosqueda-Garcia & Kunos, 1987). These results may be due to changes in opioid receptor complex formation, with a potential shift from  $\mu/\mu$  homodimers to  $\mu/\delta$  heterodimers. It would be of interest to investigate this theory, particularly if restraint and other stimuli can also produce similar effects on the composition of opioid receptor complexes. Thus, the decrease in  $\mu$ -opioid receptor density observed in the commissural NTS of WKY rats exposed to restraint may represent a change in opioid receptors from  $\mu/\mu$  homodimers to  $\mu/\delta$  heterodimers.

The majority of POMC-containing neurons in the NTS are located in the caudal region (Bronstein *et al.*, 1992). At this time, the relationship between these POMC neurons and the  $\mu$ -opioid receptors in the commissural NTS is unknown. If the chronic restraint-induced alterations in  $\mu$ -opioid receptor density do have an impact on the commissural NTS POMC-containing neurons, then the activity of the cardiovascular, respiratory and nociceptive systems may be modified accordingly, considering that a study has demonstrated that these opiocortin neurons project to nuclei including the LC, PB and RVLM (Joseph & Michael, 1988). Neurons of the commissural NTS also project to other regions, including the BNST, amygdala (Ce, Me), PVN, SON, ARC and PAG (Sim & Joseph, 1994). Moreover, the neurohypophysis also receives afferent input from the commissural NTS, suggesting that restraint-induced changes in  $\mu$ -opioid receptors in this caudal subregion of the NTS of WKY may alter the release of some posterior pituitary hormones (Garten *et al.*, 1989).

### 4.4.3 $\delta$ -OPIOID RECEPTOR AUTORADIOGRAPHY

#### 4.4.3.1 Strain comparison

A number of radioligands have been developed, characterised and used to investigate  $\delta$ -opioid receptor function and distribution, including [ $^3\text{H}$ ]-naltrindole, [ $^3\text{H}$ ]-DPDPE and [ $^3\text{H}$ ]-DSLET (Drower *et al.*, 1993; Hiller *et al.*, 1996). The latter two ligands are  $\delta$ -opioid receptor agonists, and have since been shown to label at least two different states of the  $\delta$ -opioid receptor (Hiller *et al.*, 1996). Naltrindole is a  $\delta$ -opioid receptor selective antagonist, and can inhibit the binding of both [ $^3\text{H}$ ]-DPDPE and [ $^3\text{H}$ ]-DSLET (Contreras *et al.*, 1993), suggesting that [ $^3\text{H}$ ]-naltrindole can label both the so-called  $\delta_1$  and  $\delta_2$  states. One group (Drower *et al.*, 1993) used [ $^3\text{H}$ ]-naltrindole in an autoradiographic investigation of the distribution of  $\delta$ -opioid receptors throughout the CNS and spinal cord of SD rats. They reported dense populations of [ $^3\text{H}$ ]-naltrindole binding sites in regions such as the rostral caudate nucleus, ventral medial thalamus, cortex, NAcc and OB. The present study also detected moderate to dense populations of  $\delta$ -opioid receptors in the CPu and cortex. In addition, the current investigation found that amygdaloid subregions such as the BL, BM and Me also contained a high level of  $\delta$ -opioid receptors.

Similar to others (Drower *et al.*, 1993), the present study also visualised [ $^3\text{H}$ ]-naltrindole binding sites in the hippocampus, thalamus, hypothalamus and IOC. Previous studies of the central distribution of  $\delta$ -opioid receptors using ligands other than [ $^3\text{H}$ ]-naltrindole have reported that medullary and pontine populations of  $\delta$ -opioid receptors are of low density, and often undetectable (Tempel & Zukin, 1987; Mansour *et al.*, 1993; Hiller *et al.*, 1996). The studies of Tempel (Tempel & Zukin, 1987) and Mansour (Mansour *et al.*, 1993) used the " $\delta_1$ -selective" ligand [ $^3\text{H}$ ]-DPDPE in their studies, and as demonstrated in the study by Hiller and colleagues (Hiller *et al.*, 1996), the density of the " $\delta_1$ " subtype was generally lower (<10%) than " $\delta_2$ " receptor density in the same nucleus. Therefore, the use of [ $^3\text{H}$ ]-naltrindole has resulted in a much more comprehensive report of the central distribution of  $\delta$ -opioid receptors in the rat CNS. As such, the present study was able to report and quantify additional  $\delta$ -opioid receptor populations that were located in regions such as the LC, Tcg, cerebellum, NTS, CVLM and XII.

The distribution of [ $^3\text{H}$ ]-naltrindole binding sites was found to be similar between the normotensive WKY rats and the hypertensive SHR strain. Upon closer analysis, marked

differences in the density of  $\delta$ -opioid receptors in specific regions were revealed, with a general reduction in  $\delta$ -opioid receptor density in the CNS of SHR compared to WKY. This result does not agree with the findings of Yin and colleagues (Yin *et al.*, 1996a), who reported that  $\delta$ -opioid receptor density was significantly increased in regions such as the caudate and hypothalamus in SHR compared to WKY. The study published by Yin and co-workers (Yin *et al.*, 1996a) used a much different methodological approach to label  $\delta$ -opioid receptors than the present study. Brain sections were incubated with [ $^3$ H]-etorphine, a non-selective opioid receptor ligand, with binding to the  $\mu$ - and  $\kappa$ - subtypes inhibited by concomitant incubation of ohmefentanyl ( $\mu$ ) and U69-593 ( $\kappa$ ). A protocol such as this is associated with a number of potential problems, particularly regarding the selectivity of the ligands. A previous study demonstrated that ohmefentanyl also recognised  $\sigma$  receptors (Wang *et al.*, 1991), while U69-593 appears to be selective for a particular affinity state of the  $\kappa$ -opioid receptor which is postulated to represent the  $\kappa_1$  subtype (Nock *et al.*, 1990). Thus, [ $^3$ H]-etorphine in combination with ohmefentanyl and U69-593 as blocking agents has the potential to label many different opioid receptors in addition to the  $\delta$  subtype.

Moreover, etorphine has a high affinity for the proposed  $\epsilon$ -opioid receptor that also binds  $\beta$ -endorphin (Nock *et al.*, 1990). A relatively high density of  $\epsilon$ -opioid receptors has previously been detected in the hypothalamus (see Narita & Tseng, 1998), while the present study found a relatively low level of  $\delta$ -opioid receptors throughout this region. If the protocol used by Yin and colleagues was selective for the  $\delta$ -opioid receptor, then the density of their so-called " $\delta$ -opioid receptors" in the hypothalamus should be low (Yin *et al.*, 1996a). However, a moderate to high density of " $\delta$ -opioid receptors" were detected in various hypothalamic regions, providing further evidence that they were investigating more than the  $\delta$ -opioid receptor subtype (Yin *et al.*, 1996a). It is therefore clear that the results of Yin and co-workers (Yin *et al.*, 1996a) need to be reviewed carefully, keeping in mind the use of conditions that were not optimal for  $\delta$ -subtype selectivity.

All amygdaloid subregions included in the present study, which were the BM, BL and Me, contained a significantly lower density of  $\delta$ -opioid receptors when compared to the same regions in the WKY strain. This result is in contrast to a previous report of elevated levels of binding sites for [ $^3$ H]-DPDPE in membranes of the grossly dissected amygdala (Bhargava & Rahmani, 1993). The methodological differences, such as the use of membranes instead of slide-mounted sections, may account for the discrepancy. In addition, Bhargava and colleagues used [ $^3$ H]-

DPDPE, and this ligand may be labelling a particular affinity state (or subtype) of the  $\delta$ -opioid receptor, while in contrast, current data suggest that naltrindole may recognise all  $\delta$ -opioid receptor affinity states. Although there have been studies of opioid peptide concentrations in the CNS of WKY and SHR (Bhargava *et al.*, 1988; Li *et al.*, 1992), these studies have not included the amygdala in their investigation. Thus, there is a need for detailed investigations of the functional role of amygdaloid  $\delta$ -opioid receptors, with a particular focus on their relationship with cardiovascular control, nociception and behaviour in WKY and SHR.

Another forebrain region where the density of  $\delta$ -opioid receptors were significantly reduced in SHR compared to WKY was the CPu. Previous data (including Yin *et al.*, 1996a) has demonstrated that SHR have a higher density of  $\delta$ -opioid receptors in the CPu compared to WKY and SD (Kujirai *et al.*, 1991). Kujirai and colleagues (Kujirai *et al.*, 1991) used the  $\delta_1$ -selective agonist [ $^3$ H]-DPDPE in their study, providing a possible explanation for the discrepancy in results compared to the present thesis. Adding further complexity to the status of the striatal opioid system in hypertensive rats are the results of the studies conducted by Bhargava (Bhargava *et al.*, 1988) and Li (Li *et al.*, 1992), who found that levels of Met-enkephalin-ir were similar in the CPu of SHR compared to WKY and SD. In addition, the level of prepro-ENK mRNA was not different between WKY and SHR strains, while proDYN mRNA was significantly reduced in the CPu of SHR compared to WKY (see section 3.3.2.1 and 3.3.3.1). Thus, as the processing of proDYN mRNA can yield at least 3 copies of Leu-enkephalin, reduced transcription of proDYN mRNA in the CPu may produce a concomitant suppression of striatal  $\delta$ -opioid receptors. An additional study that measures the levels of Leu-enkephalin-ir in the CPu of WKY and SHR would provide more information regarding the relationship between proDYN (and possibly prepro-ENK) mRNA and  $\delta$ -opioid receptors.

In the SHR strain,  $\delta$ -opioid receptor density was significantly reduced in the hippocampus when compared to their normotensive WKY counterparts. This finding is in contrast to the studies of Yin (Yin *et al.*, 1995) and Kujirai (Kujirai *et al.*, 1991), who reported similar levels of  $\delta$ -opioid receptor binding sites in the hippocampus and elevated [ $^3$ H]-DPDPE binding site density in the CA2 and CA3 hippocampal subregions of WKY and SHR, respectively. The reasons for these discrepancies, apart from the aforementioned methodological differences, are unclear.

Pontine regions contained similar levels of [ $^3$ H]-naltrindole binding sites in both normotensive and hypertensive strains. In the medulla, the NTS of SHR contained a

significantly lower density of  $\delta$ -opioid receptors compared to WKY. This result does not agree with (Yin *et al.*, 1995) who reported a similar level of  $\delta$ -opioid receptors between strains in this region. The study by Kujirai and co-workers (Kujirai *et al.*, 1991) did not quantify  $\delta$ -opioid receptor density in any regions located caudal to the PAG. Similarly, no evidence of strain-related differences in  $\delta$ -opioid receptor density or Met-enkephalin levels in the NTS can be gained from the work of Li (Li *et al.*, 1992) and Bhargava (Bhargava & Rahmani, 1993), as these studies measured receptor density and peptide content in the combined pons/medulla, of which the NTS constitutes a minor component.

Another medullary region where levels of  $\delta$ -opioid receptors were significantly lower in SHR compared to WKY was the CVLM. This study represents the first report of a difference in  $\delta$ -opioid receptor density in the CVLM of SHR in comparison with any normotensive strain, as the majority of previous research has focussed on the forebrain or measured the pons/medulla as a single structure (Kujirai *et al.*, 1991; Bhargava & Rahmani, 1993; Yin *et al.*, 1995). The CVLM has a primary role in cardiovascular regulation, with stimulation of neurons within the CVLM producing a direct inhibition of sympathetic vasomotor tone at the level of the RVLM (Dampney, 1994). Badoer and colleagues (Badoer & Chalmers, 1992) reported that intra-CVLM naloxone induced a hypotension and bradycardia, demonstrating that the CVLM may receive a tonic inhibitory opioid input. Further characterisation of this response was provided by Drolet and colleagues using rabbits, with intra-CVLM administration of a selective  $\delta$ -opioid receptor antagonist and Leu-enkephalin producing a depressor response (Drolet *et al.*, 1991). The underlying mechanism producing these surprisingly similar effects of  $\delta$ -opioid receptor agonists and antagonists is not known. While the cardiovascular effects of selective  $\delta$ -opioid receptor stimulation in the CVLM has not been investigated in rats, the observation that intra-CVLM administration of DAME, a non-selective  $\mu/\delta$  agonist, elicited a pressor and tachycardic response suggests that  $\delta$ -opioid receptors in the rat CVLM may inhibit the activity of CVLM depressor neurons (Willette *et al.*, 1984b). However,  $\mu$ -opioid receptors may also be mediating the cardiovascular effects of DAME in the CVLM and further experiments using more selective ligands would provide a clearer indication of the roles of these opioid receptor subtypes in the CVLM. Further research is also required to determine the functional and cardiovascular consequences of alterations in [ $^3$ H]-naltrindole binding sites in the CVLM of SHR compared to WKY.

#### 4.4.3.2 Effects of restraint stress

Brain sections obtained from rats exposed to both acute and chronic restraint stress were subjected to an autoradiography protocol that was designed to quantify stress-induced changes in  $\delta$ -opioid receptor density. Using [ $^3$ H]-naltrindole as the  $\delta$ -selective ligand, region- and strain-specific changes were observed in both normotensive (WKY) and hypertensive (SHR) strains after acute and chronic restraint. Detectable levels of  $\delta$ -opioid receptors were observed in 3 amygdaloid subregions, the Me, BL and BM. In both strains, the density of [ $^3$ H]-naltrindole binding sites in the Me and BL was not altered by either acute or chronic restraint. However, significant increases in  $\delta$ -opioid receptor density were observed after 1, 3 and 10 sessions of restraint stress in the BM of WKY, with no changes detected in the BM of SHR. Neurons within the BM, BL and Me of normotensive rats are activated by stressful stimuli such as restraint and swim stress (Chen & Herbert, 1995; Cullinan *et al.*, 1995; Krukoff & Khalili, 1997). With  $\delta$ -opioid receptors present in these three amygdaloid regions, it is not clear why the BM subnucleus has a higher sensitivity to the restraint paradigm. Furthermore, section 4.3.1.2 demonstrated that  $\mu$ -opioid receptor density did not change in the BM of either strain at any time during the restraint paradigm. Thus, this effect appears to be specific to the  $\delta$ -opioid receptor. Investigations of some of the functional effects of BM stimulation in the rat have shown that the BM has a role in the regulation of corticosterone release, locomotor activity, exploration and hippocampal activity (Plaznik, 1984; Dunn & Whitener, 1986; Ikegaya *et al.*, 1996). Moreover, the BM receives projections from central regions involved in auditory processing and autonomic regulation (LeDoux *et al.*, 1990; Bernard *et al.*, 1993; Roder & Ciriello, 1993), indicating that the BM may be a relay point acting as a junction between autonomic input and behavioural outflow during stress.

Neurons within the hypothalamic VMH can be activated by a number of different stressors such as swim stress and restraint (Cullinan *et al.*, 1995; Cullinan *et al.*, 1996). In agreement with these reports, the present study found that 3 days of the restraint paradigm produced a significant increase in [ $^3$ H]-naltrindole binding sites in the VMH of WKY, with further exposure to the restraint paradigm producing an adapted response. In contrast, no change in  $\delta$ -opioid receptor density was observed in the hypertensive strain. As described in earlier sections, the VMH contains both prepro-ENK and proDYN mRNA (sections 3.3.2 and 3.3.3). In WKY, prepro-ENK mRNA expression in the VMH was not altered by acute and chronic restraint, while acute restraint produced an initial increase in proDYN mRNA levels that was sustained for 5 days

before returning towards baseline levels. Comparison of proDYN mRNA expression and  $\delta$ -opioid receptor density in the VMH throughout the restraint paradigm suggests that a relationship between proDYN mRNA synthesis and  $\delta$ -opioid receptors may exist. Within the VMH, proDYN mRNA expression may be regulated in a negative feedback loop via the  $\delta$ -opioid receptor. Therefore, when restraint produced a sustained increase in proDYN mRNA synthesis, the levels of Leu-enkephalin may have been augmented and produced changes in the density of an autoreceptor. The increase in  $\delta$ -opioid receptor density after 3 periods may therefore represent an attempt to suppress the expression of proDYN mRNA. In effect, this may be a form of neuronal adaptation to the restraint paradigm within the VMH of WKY. In the VMH of the SHR strain, this relationship is not apparent. Although there were no restraint-induced changes in  $\delta$ -opioid receptor density, chronic restraint produced significant changes in the expression of prepro-ENK mRNA in the VMH of SHR, with increases in proDYN mRNA levels observed throughout the restraint paradigm. Whether the relationship is non-existent or dysfunctional in the VMH of SHR requires further analysis.

The CPu contained a moderate to high density of [ $^3$ H]-naltrindole binding sites in both WKY and SHR. In the normotensive WKY, restraint had no effect on  $\delta$ -opioid receptor density, while a significant decrease was observed after 5 days of the restraint paradigm in the CPu of SHR. Previous studies have reported increased levels of *c-fos* mRNA in the CPu of normotensive rats after acute swim stress or restraint (Cullinan *et al.*, 1995), which suggests that another receptor system may be involved in mediating striatal neurotransmission during exposure to these stressors. A study by Zeman and colleagues (Zeman *et al.*, 1988) measured the density of [ $^3$ H]-DADLE binding sites in rats exposed to acute (1 session) and chronic (7 and 40 consecutive sessions) immobilisation that lasted for 150 min each day. The results show that [ $^3$ H]-DADLE binding site density increased and remained elevated for each of the periods of immobilisation. While these results were attributed to alterations in  $\delta$ -opioid receptor density, DADLE is a mixed  $\mu$ -/ $\delta$ -opioid receptor agonist. It is therefore likely that changes in [ $^3$ H]-DADLE binding site density may represent changes in  $\mu$ -opioid receptors, and not the  $\delta$ -opioid receptor subtype. Moreover, the results of the present study suggest that  $\mu$ -opioid receptors may account for the observations of Zeman and co-workers (Zeman *et al.*, 1988). The stress protocol in the study of Zeman and colleagues involved immobilisation, which represents a markedly greater stressor than restraint, as the rats cannot move for a 150 min period. Considering that striatal  $\delta$ -opioid receptors have been implicated in the regulation of motor activity, inhibition of movement may produce changes in  $\delta$ -opioid receptor density in the CPu. The milder stress of restraint, where

the rats are free to move but not turn around, in conjunction with the shorter restraint period, may not have been strong enough to elicit increases in  $\delta$ -opioid receptor density in the CPu in the present study.

The implications of a significant decrease of  $-20\%$  in  $\delta$ -opioid receptor density in the CPu of SHR after 5 periods of restraint are unclear. Striatal  $\delta$ -opioid receptors have been implicated in feeding, reward, analgesia and movement (Yonehara & Clouet, 1984; Dauge *et al.*, 1988; Bakshi & Kelley, 1993; Johnson & Stellar, 1994), and further studies of the striatal opioid system, in conjunction with one or more of these physiological functions, may provide a better insight into the functional consequences of chronic restraint-induced reductions in striatal  $\delta$ -opioid receptor density.

In the cortex, a temporally different response to the restraint paradigm was observed in WKY and SHR. In WKY, a gradual increase in  $\delta$ -opioid receptor density occurred during the first 5 periods of restraint and peaked on the fifth day, with only a slight increase in  $\delta$ -opioid receptor density after 10 sessions of restraint. In SHR, 3 sessions of restraint produced a significant decrease that was followed by a gradual return to baseline levels of  $\delta$ -opioid receptor density. Despite the opposing direction of the changes in  $\delta$ -opioid receptor density, the results clearly show that cortical  $\delta$ -opioid receptors are sensitive to perturbation by the restraint paradigm. A previous report has also found that the mild stress of handling and gentle restraint, as well as footshock, produced alterations in  $\delta$ -opioid receptor binding in the Fr and Cing in SD rats, although receptor density was decreased in this study (Stein *et al.*, 1992). The functional role of  $\delta$ -opioid receptors in the cortex has not received a great deal of attention. A study by Sbrenna and colleagues suggested that  $\mu$ - and  $\kappa$ -opioid agonists have a much stronger regulatory influence on glutamatergic and GABAergic cortical neurotransmission than selective  $\delta$ -opioid receptor agonists (Sbrenna *et al.*, 1999). Thus, further studies using intra-cortical application of  $\delta$ -opioid receptor antagonists are required to determine how these opposite changes in  $\delta$ -opioid receptor levels in the cortex affect the behavioural and temporal response to restraint in WKY and SHR.

Chapter 3 (section 3.3.2.2) described markedly different changes in prepro-ENK mRNA expression in the LC of WKY and SHR rats exposed to restraint. The restraint paradigm also produced strain-specific alterations in the density of [ $^3$ H]-naltrindole binding sites in WKY and SHR strains. Specifically, 3 periods of restraint elicited an increased level of  $\delta$ -opioid receptor

density in the LC of WKY, while acute and chronic restraint had no effect on the level of  $\delta$ -opioid receptors in the SHR LC. Comparison of the prepro-ENK mRNA and  $\delta$ -opioid receptor response profile in the LC of WKY demonstrates a degree of similarity, with day 3 of the restraint paradigm producing the largest increase in gene expression and receptor density. It therefore appears that an increase in the production of prepro-ENK mRNA and the enkephalins in LC neurons may be producing an associated increase in receptor density. In contrast in SHR, there does not appear to be any correlation between prepro-ENK mRNA and  $\delta$ -opioid receptor levels.

In the LC,  $\delta$ -opioid receptor-ir has been visualised on presynaptic axonal membranes, and has also been colocalised with Met-enkephalin-ir, suggesting that  $\delta$ -opioid receptors may have an autoregulatory role in addition to a presynaptic modulation of neurotransmission (Ronken *et al.*, 1993; Van Bockstaele *et al.*, 1997). While the  $\delta$ -opioid receptor agonist DSLET had no effect on the release of catecholamines in the *in vitro* LC slice, Met-enkephalin and Leu-enkephalin have been detected in the same neurons as both GABA and glutamate (Ronken *et al.*, 1993; Van Bockstaele & Chan, 1997; Van Bockstaele *et al.*, 2000). There have been no studies of the effects of  $\delta$ -opioid receptor agonists on the release of GABA or glutamate, and further electrophysiological studies may provide stronger evidence for a synergistic action between  $\delta$ -opioid receptors and either GABA or glutamate receptors.

The LC receives enkephalin-containing afferent input from the PrH and nucleus paragigantocellularis, two medullary regions that represent the primary inputs to the LC (Aston-Jones *et al.*, 1991; Drolet *et al.*, 1992). Therefore,  $\delta$ -opioid receptors, together with the  $\mu$ -subtype which was also detected in the LC (see Table 4.2), may be in a position to modulate the dissemination of autonomic information, particularly that pertaining to the status of the cardiovascular system. If so, then the lack of changes in  $\delta$ -opioid receptor density in SHR rats exposed to the restraint paradigm may indicate a lack of sensitivity and plasticity to stressful stimuli and contribute to an altered cardiovascular stress response that has been previously observed in this strain (McDougall *et al.*, 2000).

The NTS and CVLM have an important role in cardiovascular regulation, particularly because they are components of the central baroreceptor reflex pathway (Dampney, 1994). Neither acute nor chronic restraint stress had any effect on  $\delta$ -opioid receptor density in either of these regions in WKY. However in the SHR strain, [ $^3$ H]-naltrindole binding site density was altered by restraint in both the NTS and CVLM. In the CVLM of SHR, 5 periods of restraint

produced a significant reduction in  $\delta$ -opioid receptor density. As described in the previous section (section 4.3.2.1), the exact cardiovascular effects following stimulation of the  $\delta$ -opioid receptor in the rat CVLM are unclear. As such, it is difficult to predict how changes in  $\delta$ -opioid receptor density during restraint impacts upon the cardiovascular status of SHR rats.

In the NTS of SHR, an interesting temporal response was observed during the restraint paradigm. Acute restraint produced a significant elevation in [ $^3$ H]-naltrindole binding sites in both medial and commissural NTS. This was followed by a significant reduction in  $\delta$ -opioid receptor density after 3 and 5 sessions of restraint. On the tenth day of the restraint paradigm,  $\delta$ -opioid receptor density appeared to be returning to pre-stress levels, suggesting that adaptation may be occurring. Moreover, there are two facets of the response profile that suggest that the central mechanisms at the level of the NTS that "buffer" the status of the autonomic system in SHR during both rest and exposure to stress may not be functioning at an optimum level. Firstly, restraint produced a marked increase in  $\delta$ -opioid receptor density after 1 period in SHR and not WKY, indicating that  $\delta$ -opioid receptors within the NTS of SHR are sensitive to stressful stimuli. Secondly, in an attempt to respond to the initial exposure to restraint, there appears to be an "overshoot" with  $\delta$ -opioid receptor density falling to levels well below those observed in the unstressed state. Further exposure to the restraint paradigm provides time for the  $\delta$ -opioid receptor system in the NTS of SHR to respond effectively and adapt and almost return to resting levels of  $\delta$ -opioid receptor density.

The role of  $\delta$ -opioid receptors in the NTS is unclear, but they may be implicated in cardiovascular control (see section 4.3.2.1). If so, then changes in the density of  $\delta$ -opioid receptors in the NTS of SHR during restraint may be associated with an altered cardiovascular response to the same stress paradigm that was reported in the study by McDougall and colleagues (McDougall *et al.*, 2000). However, there does not appear to be a strong correlation between the restraint-induced changes in  $\delta$ -opioid receptor density in the NTS of SHR and the cardiovascular response to restraint, suggesting that a close relationship between  $\delta$ -opioid receptors and cardiovascular control is unlikely. Furthermore, the minimal effects of  $\delta$ -opioid receptor ligands on the electrophysiological activity of NTS neurons also indicates that changes in  $\delta$ -opioid receptors, particularly decreases in  $\delta$ -opioid receptor density, may not produce marked autonomic changes during stress (Rhim *et al.*, 1993; Rhim & Miller, 1994).

Interestingly, there have been studies promoting the hypothesis of homodimers and heterodimers with  $\delta$ -opioid receptors and additional subtypes (Cvejic & Devi, 1997; Jordan & Devi, 1999). Thus, it is possible that stress-induced alterations in  $\delta$ -opioid receptor density in the NTS of SHR may reflect shifts in concentrations of particular dimers. Moreover, if the  $\delta$  heterodimers include  $\mu$  or  $\kappa$  components then autonomic functions associated with  $\mu$ - and  $\kappa$ -opioid receptors within the NTS may be adversely affected (Hassen *et al.*, 1982; Hassen *et al.*, 1984). Presently, not a great deal of research has been directed at this particular aspect of the molecular biology of the opioid receptors, and future studies will undoubtedly provide a better understanding of their prominence, distribution and function in the NTS and throughout the CNS.

#### 4.4.4 $\kappa$ -OPIOID RECEPTOR AUTORADIOGRAPHY

##### 4.4.4.1 Strain comparison

Since the first report that characterised U69-593 in 1985 (Lahti *et al.*, 1985), U69-593 has been classified as a " $\kappa_1$ "-selective ligand (Nock *et al.*, 1990). However, as there has only been one  $\kappa$ -opioid receptor that has been cloned, U69-593 will be referred to as a  $\kappa$ -opioid receptor ligand (Li *et al.*, 1993; Yakovlev *et al.*, 1995). While other  $\kappa$ -selective ligands have been developed, some autoradiographic studies have used non-selective ligands either with or without the addition of  $\mu$ - and  $\delta$ -opioid receptor blocking agents (Nock *et al.*, 1990; Mansour *et al.*, 1994b). These non-selective ligands, including [ $^3$ H]-bremazocine and [ $^3$ H]-EKC, have now been classified as non-selective opioid receptor ligands that may also label the putative " $\kappa_2/\epsilon$ "-opioid receptor and are not the optimum choice for detailed autoradiography studies of the  $\kappa$ -opioid receptor (section 1.3.1.3).

While studies have described the distribution of  $\kappa$ -opioid receptors using [ $^3$ H]-U69-593 throughout the CNS of normotensive rats (Mansour *et al.*, 1994b), there have been no autoradiographic reports of the distribution of [ $^3$ H]-U69-593 binding sites in the CNS of the hypertensive SHR. Yin and colleagues attempted to compare the levels of [ $^3$ H]-U69-593 binding sites in normotensive and hypertensive brain, but did not publish any data as the density of  $\kappa$ -opioid receptors was found to be too low (Yin *et al.*, 1996a). In addition, McConnaughey and

co-workers (McConnaughey *et al.*, 1992) compared the level of dynorphin binding sites in WKY and SHR using a membrane preparation consisting solely of hippocampal membranes. As described in the introduction (section 1.3.1.3), U69-593-sensitive-opioid receptors are not abundant in the hippocampus. The putative " $\kappa_2$ "-opioid receptor that is recognised by the benzomorphans (bremazocine and EKC) was found to be moderately distributed throughout the hippocampal formation, suggesting that the study of McConnaughey and colleagues (McConnaughey *et al.*, 1992) may be investigating a mixed population of  $\kappa$ -opioid receptors that consists primarily of the putative " $\kappa_2$ "-opioid receptor. Another investigation of central  $\kappa$ -opioid receptors in WKY and SHR incubated the non-selective ligand [ $^3$ H]-EKC in the presence of DAGO and DADLE with membranes collected from selected brain regions (Bhargava & Das, 1986). These conditions are not optimal for delineation of binding to the  $\kappa$  receptor subtype, and thus represent non- $\mu$ /non- $\delta$ -opioid binding sites that include both putative subtypes of the  $\kappa$ -opioid receptor, plus any additional opioid receptors not blocked by DAGO and DADLE. Furthermore, as the latter two studies used brain membrane preparations and not slide-mounted sections, the degree of anatomical resolution is poor. The present study, using autoradiography with a  $\kappa$ -selective ligand and brain slices through selected regions of the CNS, therefore represents the next logical step in the investigation of the  $\kappa$ -opioid receptor system in the brains of normotensive and hypertensive rats.

In both normotensive and hypertensive rats, the distribution of [ $^3$ H]-U69-593 binding sites was similar throughout the CNS. Furthermore, the distribution compared favourably with the study by Mansour and colleagues (Mansour *et al.*, 1994b), with  $\kappa$ -opioid receptor populations detected in regions such as the PVN, PVA, Me, st, Par, DEn, Teg, NTS and IOC. In the present study, quantification of the density of [ $^3$ H]-U69-593 binding sites in the CNS of WKY and SHR revealed that SHR have a globally elevated level of  $\kappa$ -opioid receptors. In all regions examined,  $\kappa$ -opioid receptor binding site density was significantly increased by at least +35% and up to +163% in SHR compared to WKY. One of the regions where the density of  $\kappa$ -opioid receptors was significantly elevated in SHR was the PVN. These data compliment earlier studies of the opioid system in the hypothalamus of hypertensive and normotensive rats. Two studies have reported significantly elevated levels of dynorphin A, dynorphin B and dynorphin A (1-13)-ir in the hypothalamus of SHR compared to WKY (Bhargava *et al.*, 1988; Tan-No *et al.*, 1997). Adding further support to these results was the study completed by Bhargava and co-workers (Bhargava & Das, 1986), who reported an increased level of [ $^3$ H]-EKC binding sites in the hypothalamus of SHR compared to WKY. These three studies analysed the entire

hypothalamus, and in conjunction with the results of the present study, they suggest that the PVN is a likely hypothalamic subregion where both dynorphin levels and  $\kappa$ -opioid receptor density is elevated in SHR compared to WKY. However, work in the previous chapter (section 3.3.3.1) demonstrated that proDYN mRNA levels were not altered in the PVN of SHR compared to WKY. A possible explanation is an increase in translation of the proDYN transcript in the PVN of SHR, so that peptide levels or receptors on terminals in the PVN are increased in SHR while the precursor mRNA concentrations are not changed.

SHR rats were also found to have an increased level of [ $^3$ H]-U69-593 binding sites in the Me when compared to their normotensive counterparts. Furthermore, there are no reports of physiological changes following  $\kappa$ -opioid receptor stimulation in the Me of either normotensive or hypertensive rats. The Me has been reported to have a role in the stress response, particularly in regards to the control of the tuberoinfundibular neurons of the pPVN, analgesia and regulation of HPA axis activity (Dunn & Whitener, 1986; Chen & Herbert, 1995; Oliveira & Prado, 1998; Dayas *et al.*, 1999), and the  $\kappa$ -opioid receptor may be involved in one or all of these processes. To gain an insight into the functional effects of elevated  $\kappa$ -opioid receptor density in SHR, further studies could compare the effects of microinjection of  $\kappa$ -opioid agonists/antagonists into the Me on nociception, activation of neurons in the pPVN and SON and the release of hormones such as CRF and corticosterone in SHR and WKY.

Both the commissural and medial subregions of the NTS in SHR contained significantly elevated levels of  $\kappa$ -opioid receptors when compared to WKY. Differences in  $\kappa$ -opioid receptor density or concentrations of endogenous  $\kappa$ -opioid receptor ligands such as dynorphin in the NTS of normotensive and hypertensive rats have not been studied. While the previous chapter (section 3.3.3.1) demonstrated that proDYN mRNA levels were reduced in the NTS of SHR compared to WKY, there have been no reports of the colocalisation of  $\kappa$ -opioid receptors and proDYN mRNA in NTS neurons. Thus, it is possible that presynaptic or postsynaptic  $\kappa$ -opioid receptors may be located on membranes that are apposed by either intrinsic dynorphin-containing NTS interneurons or afferent dynorphin input to the NTS. Furthermore, they may be located in the NTS in a position to modulate the release of other neurotransmitters or dynorphin via a negative feedback mechanism. Until more information is gained in this respect, it is unclear how proDYN mRNA levels are related to  $\kappa$ -opioid receptor density in the NTS. However, the observation that proDYN mRNA is significantly reduced in the NTS of SHR, while  $\kappa$ -opioid receptor levels are increased in the SHR NTS compared to WKY, indicates that

$\kappa$ -opioid receptors in the NTS may not function strictly as negative feedback modulators of dynorphin release.

#### 4.4.4.2 Effects of restraint stress

To compliment sections 4.4.2.2 and 4.3.2.2, the effects of a 10 day restraint paradigm on [ $^3$ H]-U69-593 binding site density was quantified in both normotensive and hypertensive rats. In the PVN of WKY rats, acute and chronic restraint did not produce any significant alterations in  $\kappa$ -opioid receptor density. In contrast,  $\kappa$ -opioid receptors in the PVN of SHR were sensitive to acute and moderately chronic restraint stress. After 1 day of the restraint paradigm, a significant reduction in the density of [ $^3$ H]-U69-593 binding sites in the PVN of SHR was observed. Further exposure to the restraint stimulus also produced similar significant decreases in  $\kappa$ -opioid receptor binding sites in the PVN of SHR, although the magnitude of these reductions were not as large as that of the first period of restraint. On the final day of the restraint paradigm,  $\kappa$ -opioid receptor density was not altered in the PVN of SHR, suggesting the presence of an adaptive response.

Peripheral administration of  $\kappa$ -opioid receptor agonists such as MR-2034 and U50-488H can stimulate the HPA axis (Iyengar *et al.*, 1986; Calogero *et al.*, 1996), while Laorden and colleagues (Laorden *et al.*, 2000) demonstrated that peripheral administration of the  $\kappa$ -opioid agonist U50-488H produced an increase in Fos-ir in the pPVN, where the tuberoinfundibular neurons that contain CRF, the potent ACTH secretagogue are localised (Sawchenko & Swanson, 1983). These studies suggest that  $\kappa$ -opioid receptors in the PVN can modulate HPA axis activity. In addition, stressors such as i.p. hypertonic saline and exposure to a novel environment can stimulate the production of  $\kappa$ -opioid receptor mRNA in the medial pPVN, but not the lateral pPVN of normotensive rats (Yukhananov & Handa, 1996), suggesting that the  $\kappa$ -opioid receptor system in the PVN may be modulating the HPA axis during stress. However, Cover and colleagues (Cover & Buckingham, 1989) showed that intra-PVN microinjection of the  $\kappa$ -opioid receptor antagonist MR2266 potentiated the increase in plasma levels of ACTH and corticosterone induced by surgical trauma. MR2266 has also been shown to have a high affinity for the U69-593-insensitive  $\kappa$ -opioid receptor, which may be the putative  $\epsilon$ -opioid receptor that binds  $\beta$ -endorphin (Nock *et al.*, 1993). It is therefore possible that the potentiation of HPA axis activity observed following intra-PVN microinjection of MR2266 may be mediated by this

putative  $\epsilon$ -opioid receptor, as i.c.v. administration of  $\beta$ -endorphin suppresses the release of CRF and ACTH (Suda *et al.*, 1992). While  $\beta$ -endorphin could be producing these effects through any region in the CNS, a projection from the ARC to the PVN has been shown to contain ACTH-ir, and consequently,  $\beta$ -endorphin (Sawchenko *et al.*, 1982). Nevertheless, the  $\kappa$ -opioid receptor system appears to be involved in the modulation of HPA axis activity during the basal and stressed state.

In the normotensive WKY strain,  $\kappa$ -opioid receptor density was not altered by restraint, indicating that stress-induced changes in levels of the  $\kappa$ -opioid receptor are either non-existent or return to basal levels within the 60 min restraint period. In contrast in SHR, a significantly decreased  $\kappa$ -opioid receptor density was observed in the PVN during the first 5 periods of restraint, with adaptation evident after 10 days of the restraint paradigm. Thus, it appears that  $\kappa$ -opioid receptors in the PVN are implicated in the neural stress response in SHR, but not WKY. Moreover, if  $\kappa$ -opioid receptor activation in the PVN of SHR produced a similar increase in activity of the HPA axis as previously observed in WKY (Iyengar *et al.*, 1986; Calogero *et al.*, 1996; Laorden *et al.*, 2000), then the restraint-induced decrease in  $\kappa$ -opioid receptor density observed in the present thesis would be expected to produce a decrease in plasma levels of ACTH and corticosterone in SHR during exposure to restraint. In SHR, however, there have been contrasting results regarding the plasma levels of ACTH and corticosterone during stress when compared to normotensive controls (see section 1.4.4). Further studies are therefore required to determine the plasma levels of corticosterone and/or ACTH of SHR during a 10 day restraint paradigm similar to the present study, and how these levels correlate with  $\kappa$ -opioid receptor density in the PVN of SHR during restraint.

In the Me, both WKY and SHR strains exhibited a similar response to the restraint paradigm, although significant changes were only observed in the SHR strain. In SHR, the entire restraint paradigm produced a sustained significant reduction in  $\kappa$ -opioid receptor density of approximately -30%. The Me has been implicated by numerous studies in the central response to stress, with *c-fos* mRNA and Fos-ir detected in this amygdaloid subregion following restraint and other stressors (Cullinan *et al.*, 1995; Dayas *et al.*, 1999). One of these reports demonstrated that there is a stronger expression of Fos-ir in the Me than the Ce following restraint. Furthermore, the same study also showed that lesion of the Me suppressed restraint-induced expression of Fos-ir in cells located in the medial pPVN and SON, leading the authors to suggest

that the Me may be more important than the Ce in some facets of the neural, and possibly neuroendocrine response to psychological stressors (Dayas *et al.*, 1999).

While the functional effects following intra-Me administration of selective  $\kappa$ -opioid agonists or antagonists have not been completely investigated, the Me has been implicated in the regulation of a variety of physiological systems. Lesions of the Me have produced alterations in behaviour and stress-induced analgesia, as well as restraint-induced Fos expression in the hypothalamus (Shibata *et al.*, 1982; Bellgowan & Helmstetter, 1996; Dayas *et al.*, 1999). Antinociception and elevated HPA axis activity have also been observed following electrical stimulation of the Me (Dunn & Whitener, 1986; Oliveira & Prado, 1998). Thus,  $\kappa$ -opioid receptors may be able to modulate neurons within the Me that are involved in some or all of these functions, both during the resting and stressed state. Consequently, there is a need for more experiments that specifically investigate the physiological role of opioid receptors in the Me of both SHR and WKY rats.

The NTS was one of the few regions in the pons and medulla where [ $^3$ H]-U69-593 binding sites were quantified. In both strains, the NTS was subdivided into the medial and commissural components, and a slightly elevated basal level of  $\kappa$ -opioid receptors was detected in the medial NTS in both WKY and SHR, although there was no significant difference in either strain. This finding partly corresponds with an immunohistochemical study of the prevalence of dynorphin A-ir fibres in the subregions of the NTS, with the highest density of fibres localised in the medial NTS (Fodor *et al.*, 1990). Comparison of the density of  $\kappa$ -opioid receptors in the NTS at various stages during the restraint paradigm revealed some interesting differences between strains and the subregions of the NTS. Firstly, in the commissural NTS of WKY, acute and chronic restraint did not produce any significant alterations in [ $^3$ H]-U69-593 binding site density. However, in the medial NTS in the WKY strain, 3 sessions of restraint elicited a significant decrease in the density of  $\kappa$ -opioid receptors. The temporal pattern in the medial NTS of WKY was bell-shaped, with a non-significant decrease in  $\kappa$ -opioid receptor density after 1 and 5 sessions of restraint. This pattern indicates that the  $\kappa$ -opioid receptor system located in the NTS of WKY may be involved in the chronic stress response and associated coping mechanisms.

In the NTS of SHR, the response to restraint was dissimilar to that observed in WKY. While no changes were observed in the medial NTS of SHR at any time during the restraint paradigm, the commissural NTS was sensitive to chronic restraint. Acute (1 period) restraint produced non-significant elevations in  $\kappa$ -opioid receptor density, while 5 sessions of restraint produced the

largest and only significant increase in the density of [ $^3\text{H}$ ]-U69-593 binding sites. After 10 days of the restraint paradigm, the density of  $\kappa$ -opioid receptors was starting to return to baseline levels, suggesting that this decreasing trend may continue if the restraint paradigm was extended. As such, it appears that the population of [ $^3\text{H}$ ]-U69-593 binding sites in the commissural NTS of SHR exhibits a degree of plasticity that facilitates dynamic changes in density that assists in adaptation to chronic restraint.

The functional effects resulting from changes in  $\kappa$ -opioid receptor density in the NTS of both strains are unclear. Furthermore, it is not known whether the differential changes in the subregions of the NTS in each strain represent diverse roles for  $\kappa$ -opioid receptors or a simple difference in the neuronal network present within the NTS. Presently, there have been no studies comparing the functional effects following stimulation of  $\kappa$ -opioid receptors in the medial and commissural subregions of the NTS in normotensive or hypertensive strains. However, there have been a limited number of studies that have injected selective and non-selective  $\kappa$ -agonists into the NTS and observed some functional changes. Thus, intra-NTS administration of bremazocine and dynorphin in anaesthetised rats decreases HR and BP (Hassen *et al.*, 1984). Negative results have also been published, with no changes in either feeding or the cardiovascular response to haemorrhage observed after microinjection of  $\kappa$ -opioid agonists such as dynorphin A (1-17) and dynorphin A (1-13) into the NTS (Fan & McIntosh, 1993a; Kotz *et al.*, 1997). Despite the paucity of studies of  $\kappa$ -opioid receptors in the NTS and considering that vagal afferents to the NTS have been observed in close proximity to dynorphin-containing neurons in the NTS (Fodor *et al.*, 1990), further research may produce stronger evidence describing a role for  $\kappa$ -opioid receptors in the modulation of various visceral functions.

The medial and commissural NTS have slightly different functions according to their afferent and efferent connections. Terminals from a variety of peripheral sensory receptors are topographically distributed throughout the NTS, with the medial and commissural NTS receiving the majority of these visceral inputs. While the terminal fields of some receptors encompass both the medial and commissural NTS, others are predominantly found in one of these NTS subregions (see Barraco *et al.*, 1992). Moreover, the efferent projections of the medial and commissural NTS are related to the afferent input from the periphery and from higher brain centres that serve to modulate the peripheral sensory input. Thus, the results of the present study may also be associated with the sometimes subtle differences in function of the medial and commissural NTS. Microinjection techniques presently available to researchers are adequately

equipped to investigate the effects of application of various agonists and antagonists into discrete subdivisions of the NTS. Thus microinjection of selective  $\kappa$ -opioid receptor agonists into the medial and commissural NTS, coupled with observation of nociception and gastrointestinal, respiratory and cardiovascular parameters, would provide a clearer delineation of the effects of stimulation of  $\kappa$ -opioid receptors in different regions of the rat NTS.

Published evidence of functional effects attributed to stimulation of  $\kappa$ -opioid receptors in specific regions other than the PVN, SON and NTS is scarce. Thus, it is difficult to predict how changes in many of the regions where [ $^3\text{H}$ ]-U69-593 binding site density was elevated in the unstressed and restrained (stressed) SHR compared to WKY influences physiological and autonomic functions. These regions include the Me, PVA, DEN and Teg, with some of these implicated in the modulation of autonomic processes, including analgesia and the stress response (Oliveira & Prado, 1998; Bubser & Deutch, 1999; Emmert & Herman, 1999). Considering that SHR and WKY rats exhibit phenotypical differences in the stress response and nociception, contributions from elevated  $\kappa$ -opioid receptor density in brain regions such as the Me and PVA cannot be discounted until detailed studies are completed in the future.

#### 4.4.5 GAL RECEPTOR AUTORADIOGRAPHY

##### 4.4.5.1 Strain comparison

The distribution of [ $^{125}\text{I}$ ]-GAL binding sites was visualised through various levels of the rat CNS, including the forebrain at the level of the PVN and amygdala, pons at the level of the LC and PB and caudal medulla at the level of the NTS. The distribution of [ $^{125}\text{I}$ ]-GAL binding sites matches previous autoradiographic studies using the same ligand (Skofitsch *et al.*, 1986; Melander *et al.*, 1988), with binding detected in nuclei such as the Ce, Me, VMH, PB and NTS.

In the present study, the distribution of [ $^{125}\text{I}$ ]-GAL binding sites in the forebrain, pons and medulla of both WKY and SHR rats were similar. However, significant differences in the density of [ $^{125}\text{I}$ ]-GAL binding sites in a number of forebrain regions were detected between WKY and SHR. SHR rats were found to have a significantly decreased density of GAL receptors in the Ce, BM and VMH, and a significantly increased density of [ $^{125}\text{I}$ ]-GAL binding sites in the CL. While studies investigating the role of the CL and BM in the maintenance or

development of hypertension have been limited, the Ce has been the focus of a number of studies designed to determine the role of this amygdaloid nucleus in the development of hypertension. Significant decreases in GABA<sub>A</sub> receptors (Kunkler & Hwang, 1995) and dynorphin (1-8)-ir (Feuerstein *et al.*, 1983) have been reported in the Ce of SHR; however, electrical stimulation of the Ce in the SHR does not elicit exaggerated cardiovascular responses (Galeno & Brody, 1983) when compared with normotensive controls. Lesions of the Ce have been shown to attenuate the development of hypertension in SHR (Folkow *et al.*, 1982; Sharma & Gelsema, 1995), possibly due to a decreased weight gain in the lesioned rats (Sharma & Gelsema, 1995). Interestingly, microinjection of GAL into the Ce can modulate food intake (Corwin *et al.*, 1993), but the reduced density of GAL receptors in the Ce of SHR reported in this study was not translated into any changes in weight in the SHR rats.

This study also reported a decrease in [<sup>125</sup>I]-GAL binding sites in the VMH of SHR, a hypothalamic region where administration of GAL can affect feeding (Schick *et al.*, 1993). Functional data pertaining to GAL at the level of the VMH are scarce. However, neurochemical alterations have been detected within this hypothalamic nucleus in the hypertensive state, with intra-VMH administration of baclofen (GABA<sub>B</sub> agonist) producing significantly different changes in BP, HR and sympathetic nerve activity in SHR compared to WKY (Takenaka *et al.*, 1996). Thus, the VMH has the capacity to alter sympathetic outflow and cardiovascular parameters, and future studies investigating and comparing the functional role of GAL within the VMH of WKY and SHR, will determine whether GAL contributes to any of the phenotypic variations observed in SHR.

Furthermore, it is clear that research into GAL and its receptors is still in its infancy. Following the characterisation of each of the receptor subtypes and subsequent synthesis of selective ligands, functional, electrophysiological and neurochemical studies will provide a greater knowledge of the modulatory roles of GAL in the CNS. Once these have been completed, comparisons of the GAL system in diseased states such as the SHR model of genetic hypertension will assist in an understanding of the neural bases of these disorders.

#### 4.4.5.2 Effects of restraint stress

GAL has been associated with the neural and neuroendocrine response to a number of stressors, including restraint (section 3.3.4.2), ether inhalation and cold stress (Malendowicz *et*

*al.*, 1994). There have been no reports describing changes in GAL receptor density following exposure to stress, and as such, the present study provides the first investigation of the effect of stress (restraint) on [<sup>125</sup>I]-GAL binding sites in the rat CNS. Exposure to restraint stress produced significant changes in a variety of CNS nuclei in both normotensive and hypertensive rats. Statistical comparison of the pattern of the response to restraint stress between strains identified a number of regions where the response of neurons containing [<sup>125</sup>I]-GAL binding sites was similar in each strain. Restraint stress was found to elicit changes in some, but not all, of these regions. Subregions of the thalamus, the raphe nuclei and the NTS were some of the regions in both strains where the level of [<sup>125</sup>I]-GAL binding site density was maintained at basal levels throughout the restraint stress paradigm, demonstrating that the restraint-induced alterations in GAL receptor density were region-specific.

There appeared to be no strain dependent difference in the temporal response of [<sup>125</sup>I]-GAL binding sites in the Me to restraint. However, while there were no significant alterations in [<sup>125</sup>I]-GAL binding site density in the Me of SHR after all periods of restraint, a significant decrease of ~ -40% was observed after one period of restraint stress in WKY. The Me can be activated by exposure to restraint stress, and some studies have reported a higher expression of *c-fos* mRNA or Fos-ir in the Me compared to the Ce (Chen & Herbert, 1995; Bhatnagar & Dallman, 1998; Dayas *et al.*, 1999). One study also demonstrated that lesion of the Me, but not the Ce, attenuates neuronal activation in the PVN and SON, two forebrain regions that can modulate the neuroendocrine response to stress (Dayas *et al.*, 1999). In the Me, GAL-R1 mRNA and GAL are colocalised with vasopressin (Miller *et al.*, 1993), and vasopressin-containing neurons within the Me have been shown to project to forebrain regions involved in memory, such as the ventral hippocampus (Caffe *et al.*, 1987). GAL is further implicated in the modulation of memory processes since studies have shown that GAL inhibits the release of ACh in ventral hippocampal slices (Fisone *et al.*, 1987). Therefore, an intact and fully functional GAL system in the Me may be necessary for learning and memory, and an impaired capacity to learn may modify the response to chronic restraint stress. This hypothesis is reinforced by the results of (McDougall *et al.*, 2000), where SHR rats achieved a level of cardiovascular adaptation to restraint comparable to WKY only after 7 days of the restraint paradigm.

In both WKY and SHR, acute restraint stress elicited a significant decrease in [<sup>125</sup>I]-GAL binding in the Pir, a region that has a prominent role in the processing of olfactory information (Scott *et al.*, 1980; Staubli *et al.*, 1987). The Pir is sensitive to perturbation by stressors such as restraint (Cullinan *et al.*, 1995; Del Bel *et al.*, 1998), as well as immobilisation (Imaki *et al.*,

1993), footshock (Beck & Fibiger, 1995) and saline injection (Sharp *et al.*, 1991). Moreover, the fact that i.c.v. injection of CRF (Imaki *et al.*, 1993) induces expression of *c-fos* mRNA in the Pir suggests that priming and activation of olfactory processing is an integral component of the stress response.

GAL receptors in the PB of both strains were sensitive to restraint. In the lateral PB of SHR, the density of [<sup>125</sup>I]-GAL binding sites decreased following 1 and 3 periods of restraint, significantly decreased after 5 sessions, and appeared to return to baseline levels following 10 days of the restraint paradigm. A similar pattern following restraint was observed in WKY, with the largest decrease in [<sup>125</sup>I]-GAL binding site density detected after 5 periods of restraint. These results suggest that GAL receptors in the PB may be preferentially activated by chronic restraint, rather than acute exposure, and GAL receptor-containing neurons in the PB of both strains may adapt to repeated restraint stress, presumably due to altered synaptic input. The lateral PB is sensitive to activation by a variety of stressors, with Fos expression increasing in the lateral PB following exposure to hypercapnia and hypoxia, restraint and cold stress (Krukoff & Khalili, 1997; Teppema *et al.*, 1997; Bhatnagar & Dallman, 1998). The lateral PB is known to be involved in the modulation of many autonomic functions, including respiratory and cardiovascular control (Bohmer *et al.*, 1990; Lara *et al.*, 1994). Moreover, the PB is the major relay between the NTS and the forebrain for ascending visceral afferent information. As such, the PB is anatomically connected to many brain regions implicated in the stress response, including the PVN, Ce, NTS and RVLM (Moga & Gray, 1985; Moga *et al.*, 1990a; Krukoff *et al.*, 1992; Krukoff *et al.*, 1993; Larsen & Mikkelsen, 1995; Jhamandas *et al.*, 1996). While there have been limited studies of the functional effects of GAL in the PB, immunohistochemical investigations have detected GAL-ir in PB afferents from the NTS (Herbert & Saper, 1990; Krukoff *et al.*, 1992; Krukoff *et al.*, 1993), and efferents connecting the PB with the Ce project to GAL neuronal perikarya (Jhamandas *et al.*, 1996). Therefore, while the anatomical association of GAL with the PB is well established, further studies are required to clarify the functional role(s) of GAL in this nucleus.

While restraint had no effect on [<sup>125</sup>I]-GAL binding sites in the XII of WKY rats, restraint exposure for 1, 5 and 10 sessions induced significant decreases in [<sup>125</sup>I]-GAL binding site density in the XII of SHR. There was also a decrease observed after 3 periods of restraint that was not significant. The XII has not been viewed as a primary mediator in the neural response to stress, but studies in the rat have shown that it can be activated by restraint (Cullinan *et al.*, 1995). The XII has been implicated in respiration (Bach & Mitchell, 1998) and XII neurons are sensitive to

hypercapnia and hypoxia (Mazzone *et al.*, 1997; Bach & Mitchell, 1998), suggesting that GAL receptors in the XII may be implicated in stress-induced changes in respiration.

Statistical comparison of the temporal pattern of the response to restraint stress highlighted nuclei where the neurons containing GAL receptors exhibited a response to restraint that was significantly different between strains. These nuclei included the Ce, BM, VMH and Sp5C. Interestingly, basal GAL receptor density was significantly decreased in the Ce, BM and VMH of SHR compared to WKY, suggesting that an underlying neurochemical deficit may predispose the SHR to an atypical stress response in these regions. Whether the significant differences in response to restraint observed in these nuclei in SHR are associated with the impaired cardiovascular response to stress previously observed in hypertensive rats remains to be elucidated (McDougail *et al.*, 2000).

In WKY, acute restraint induced a significant decrease in the density of [<sup>125</sup>I]-GAL binding sites in the Ce, BM and VMH of approximately -40%. In all of these nuclei, as well as the Me and Pir of WKY and the Pir in SHR, increasing exposure to restraint also resulted in decreases in GAL receptor density, but this reduction was not of the same magnitude as that observed after 1 period of restraint. The density of GAL receptors in these nuclei had almost returned to baseline levels after the full 10 day restraint paradigm, suggesting that the neurons possessing GAL receptors in these nuclei were receiving altered inputs during the time course of the stress paradigm.

The previous chapter (section 3.3.4.2) reported a large degree of plasticity in the GAL system within the Ce of normotensive WKY, with an initial increase in prepro-GAL mRNA expression observed in the Ce after 1 session of restraint that returned to basal levels of expression following further exposure to restraint. A closer comparison of the two studies reveals that the changes in prepro-GAL mRNA expression (+35%) (section 3.3.4.2) and [<sup>125</sup>I]-GAL receptor density (-41%) following 1 period of restraint are both similar and opposite in direction. These results suggest that an increase in expression of prepro-GAL mRNA, and hence a possible increase in GAL peptide levels, may result in a rapid reduction in receptor number as a consequence of the restraint-induced increase in GAL peptide. The resultant parallel response to chronic restraint in both prepro-GAL mRNA and GAL receptors suggests that an intimate dynamic association between peptide synthesis and receptor density may be operative within the Ce.

The current study demonstrated that in the amygdala of SHR, particularly the Ce and BM, there were no significant alterations in [ $^{125}$ I]-GAL binding site density following acute or chronic restraint. In contrast, restraint stress induced marked increases in the expression of prepro-GAL mRNA in the Ce of SHR of up to +300%, with no adaptation observed after chronic (10 periods) periods of exposure (section 3.3.4.2). Taking the results of both studies into consideration, it appears that the increase in GAL peptide levels in the Ce of SHR following exposure to restraint does not produce any subsequent alterations in the density of GAL receptors in the same nucleus. Therefore, a comparison of the GAL system in the Ce of WKY and SHR suggests that there may be a differential relationship between GAL receptors and prepro-GAL mRNA expression in the Ce of SHR when the rats are exposed to restraint.

The Ce is an integral component of the multi-faceted response to restraint and other stressors. Lesions of the Ce have been shown to attenuate restraint-induced ACTH release (Marcilhac & Siaud, 1996), attenuate the stress-induced pressor response (Sanders *et al.*, 1994), reduce noradrenergic activity in the hypothalamus following restraint (Beaulieu *et al.*, 1987) and block the behavioural response to fearful stimuli (LeDoux *et al.*, 1988). Furthermore, a study by Roozendaal and colleagues suggests that the Ce has a significant role in passive coping following exposure to a stressful event (Roozendaal *et al.*, 1997). There are a paucity of data describing the role of GAL in the stress response of the Ce, with only one study demonstrating that GAL receptor stimulation within the Ce can modulate fear and anxiety (Moller *et al.*, 1999). With the ability of the Ce to modulate many components of the stress response, the lack of significant changes in [ $^{125}$ I]-GAL receptor density in the Ce of SHR following restraint may contribute to the altered stress response previously observed in this hypertensive strain (Li *et al.*, 1997; McDougall *et al.*, 2000).

Restraint stress induced quite different alterations in GAL receptor density in the VMH of WKY and SHR. In SHR, a significant increase was seen after 5 sessions of restraint, while GAL receptor density decreased after 1 day of exposure to the restraint paradigm in WKY. Direct activation of GAL receptors in the VMH has been shown to increase feeding in rats (Schick *et al.*, 1993); however, there have no other direct studies of GAL in this hypothalamic nucleus. Increased *c-fos* mRNA and Fos-ir have been detected following air-puff startle or footshock (Beck & Fibiger, 1995; Palmer & Printz, 1999); however, restraint or swim stress did not induce *c-fos* mRNA expression in the VMH (Cullinan *et al.*, 1995). In addition, a recent study demonstrated that the VMH is involved in the pressor response mediated by the Ce following prolonged emotional stress (Wu *et al.*, 1999).

In the Sp5C in the SHR medulla, restraint stress induced a significantly different temporal response compared to WKY. While exposure to restraint for 1, 5 and 10 periods induced a significant decrease in [ $^{125}$ I]-GAL binding site density in the Sp5C of SHR, there was a decrease in GAL receptor density in the Sp5C of SHR after 3 periods of restraint that was not significant. As with the XII in SHR, it is possible that this may represent a biphasic response, where XII and Sp5C neurons containing GAL receptors may overcome the initial acute exposure to restraint and recover with a return towards normal levels of GAL receptor density. However, as the number of restraint sessions increase, a second significant reduction in GAL receptor density follows that is maintained for 5 and 10 sessions of restraint.

Interestingly, the restraint-induced decrease in GAL receptors within the Sp5C of SHR does not appear to be returning towards control levels after 10 days of the restraint paradigm. While the adaptive response to restraint may be dysfunctional in SHR, it is also possible that GAL receptors may return to normal levels after a period of restraint that is greater than 10 days. Consequently, further experiments where the restraint paradigm lasts longer than 10 days should be completed to clarify the temporal response pattern to restraint in the Sp5C of SHR. The implications of changes in GAL receptor density in the Sp5C are not known, as the role of GAL in the Sp5C has not been reported. The Sp5C has not been a major target in the study of the neural effects of stress, but studies have demonstrated that this nucleus is sensitive to perturbation by restraint (Cullinan *et al.*, 1995). Reports suggest that the Sp5C may be involved in stress pathways activated by stressful events that elicit rapid, reflex reactions, such as craniofacial nociception and the reflex pressor response following noxious stimulation (Allen & Pronych, 1997).

The temporal response pattern observed in the SHR in both the XII and Sp5C nuclei are extremely similar, suggesting that these nuclei may be innervated by afferent neurons from the same central origin(s). The identity of these region(s) are unclear, and tracing studies where a retrograde tracer is injected into both nuclei would provide further insights into the afferent projections to the Sp5C and XII. These tracing studies should investigate the possible involvement of central regions such as the PAG where prepro-GAL mRNA has been detected (Ryan & Gundlach, 1996), as well as the peripheral ganglia of cranial and sensory nerves.

Although the present study has demonstrated that central GAL receptors are sensitive to perturbation by acute and chronic restraint, the influence of each of the GAL receptor subtypes is difficult to delineate. There are no autoradiographic studies of the distribution of each GAL

receptor subtype in the CNS due to the lack of subtype-specific GAL receptor ligands in use, other than the general GAL receptor ligand [ $^{125}$ I]-GAL. Alternatively, parallels can be drawn between the presence of GAL receptor subtype mRNA in nuclei where specific changes were observed after restraint in the present study. The presence of GAL-R1 mRNA has been reported in the Pir, Me, VMH, thalamus, lateral PB and Sp5C (Gustafson *et al.*, 1996; O'Donnell *et al.*, 1999), while expression of GAL-R2 mRNA was detected in regions such as the VMH, lateral PB and XII (O'Donnell *et al.*, 1999). A comparison is harder to describe for GAL-R3 mRNA, as the distribution of GAL-R3 mRNA was measured in whole brain regions such as the amygdala and hypothalamus, rather than a region-specific distribution such as those for GAL-R1 and GAL-R2 (Waters & Krause, 2000). The above comparison for GAL-R1 and GAL-R2 suggests that neither GAL receptor subtype is preferentially involved in the response to restraint. While the mRNA for a GAL receptor subtype might be detected in the same region as GAL binding sites, the transport of receptors from the neuron in one region to a terminal field in another cannot be discounted. Therefore, the parallels described above can only be confirmed after the completion of autoradiography experiments using conditions designed to distinguish between the different GAL receptor subtypes. The capacity to fulfill these experiments awaits the development of subtype-specific tools such as anti-receptor antibodies or selective GAL receptor agonists/antagonists.

#### 4.4.6 CONCLUSION

The present chapter used a variety of autoradiographic techniques to visualise and compare the distribution and density of the three main subtypes of opioid receptor ( $\mu$ ,  $\delta$  and  $\kappa$ ) and the GAL receptor. Using the appropriate ligands and binding conditions, a specific topographic distribution was observed for each of the 4 receptors throughout the CNS of both WKY and SHR. In particular, the distribution profile of the 3 opioid receptor subtypes was quite distinct throughout all levels of the CNS in each strain. Comparison of basal levels of receptor density for each ligand in the CNS of WKY and SHR revealed the presence of significant differences in a number of regions in the forebrain, pons and medulla oblongata that were specific for each receptor. Notably, a general decrease in [ $^3$ H]-naltrindole ( $\delta$ ) binding sites was detected in the CNS of SHR, while a global increase in the density of [ $^3$ H]-U69-593 ( $\kappa$ ) binding sites was observed in the CNS of SHR compared to WKY. Furthermore, the neural location of some of

these significant changes in receptor density in SHR may affect the modulation of various physiological functions, such as the PVN ( $\kappa$ ), amygdala ( $\mu$ ,  $\delta$ ,  $\kappa$ , GAL), NTS ( $\delta$ ,  $\kappa$ ) and CVLM ( $\delta$ ). Thus, these data provide further evidence of alterations in the neurochemistry of the SHR strain compared to WKY, and represent possible targets for future studies of impaired or atypical functional systems in SHR.

Acute and chronic restraint stress produced significant region- and strain-specific changes in binding site density in each of these receptor systems. Analysis of the temporal response to restraint revealed that in specific regions, the level of binding sites for a particular ligand at the initial acute stage of the restraint paradigm was markedly different from the density of binding sites after exposure to repeated restraint. In some cases, such as the Ce (GAL) and Me (GAL) of WKY, an initial response to acute restraint was followed by a graded return to basal levels of receptor density as the number of restraint sessions increased. Data such as these provide evidence of plasticity in a particular receptor system during exposure to a stressor such as restraint. In contrast, there were other regions where a significant change in receptor density was maintained for the restraint paradigm. Examples of this temporal response pattern were observed in the VMH ( $\delta$ ) of WKY and in the Me ( $\kappa$ ) and XII (GAL) of SHR. These results clearly demonstrate that the present restraint paradigm has the capacity to produce changes in receptor density that was specific to each receptor system. Furthermore, the observation of significantly different temporal responses to the restraint paradigm between WKY and SHR provide further evidence of an altered stress response in the hypertensive SHR strain.

## 4.5 ADDENDUM

### 4.5.1 ARE [<sup>125</sup>I]-GAL BINDING SITES LOCALISED IN THE HUMAN INFERIOR VAGAL (NODOSE) GANGLION?

The neurochemistry of the nodose ganglion has been studied using a variety of techniques in species such as the rat and human (Calingasan & Ritter, 1992; Lawrence & Jarrott, 1994; McLean *et al.*, 1997; Ashworth-Preece *et al.*, 1998). GAL has been detected within the nodose ganglion of the rat and pig (Philippe *et al.*, 1990; Calingasan & Ritter, 1992), but not in the human. In the rat, there is evidence that GAL may be implicated in the modulation of vagal transmission at the level of the NTS, the central site of termination of the afferent fibres of the nodose ganglion (Van Giersbergen *et al.*, 1992). For example, GAL-ir terminals and receptors have been detected in the rat NTS (Melander *et al.*, 1986a; Melander *et al.*, 1986b; Härfstrand *et al.*, 1987), and microinjection of GAL into the anaesthetised rat NTS modulates BP and HR (Chen *et al.*, 1996a; Shih *et al.*, 1996).

However, there have been no studies that have reported the presence of GAL receptors in the nodose ganglion. Therefore, as an additional study, *in vitro* receptor autoradiography using [<sup>125</sup>I]-GAL was utilised to determine whether [<sup>125</sup>I]-GAL binding sites exist within human nodose ganglia.

### 4.5.2 METHODS

The Ethics and Integrity in Research Committees of both Monash University and the Victorian Institute of Forensic Pathology (VIFP) granted ethical permission for this study. Human inferior vagal ganglia were obtained from cadavers at the VIFP. Donors (n=4) were male and aged between 18 and 77 years of age. For each donor, there was no previous history of neurological disease and the delay between death and autopsy was either 48 hours (3 donors) or 5 days (1 donor). Immediately after dissection, nodose (inferior vagal) ganglia from the right hand side from all cadavers were frozen over liquid nitrogen, transported on dry ice to the laboratory and stored at -80°C until further use.

For visualisation of [ $^{125}$ I]-GAL binding sites, human nodose ganglia were sectioned longitudinally on a cryostat at  $-16^{\circ}\text{C}$ . Tissue sections ( $14\mu\text{m}$ ) were thaw-mounted onto gel-chrome alum-coated microscope slides and stored at  $-80^{\circ}\text{C}$  until further use. The procedure for [ $^{125}$ I]-GAL receptor autoradiography was the same as that described in section 4.2.4. When dry, slides were apposed to X-ray film for 1 day with standard [ $^{14}\text{C}$ ] microscales and X-ray films were subsequently developed using an automatic X-ray Film Processor.

Microscope slides were then apposed to coverslips that had been previously dipped in photographic emulsion (section 2.8.2) for 4 days at  $4^{\circ}\text{C}$ . After this period, slides were processed according to section 2.9.2 and 2.10. Individual sections were then examined under a light microscope under both dark- and bright-field condensers. Using the MCID analysis system (section 2.12), specific binding for each subject ( $n=4$  subjects) was quantified and calculated by averaging the data for each nodose section ( $N=4$  sections per subject). Group data for specific [ $^{125}$ I]-GAL binding was then expressed as the mean  $\text{DPM}/\text{mm}^2 \pm \text{S.E.M.}$  for  $n=4$  subjects.

### 4.5.3 RESULTS

*In vitro* receptor autoradiography was used to visualise the distribution of [ $^{125}$ I]-GAL binding sites on sections of human nodose ganglia. A punctate distribution of [ $^{125}$ I]-GAL binding sites was found throughout the entire section of the nodose ganglion (Figure 4.18A) and specific binding was measured as  $28 \pm 2 \text{ DPM}/\text{mm}^2$  per subject ( $n=4$  subjects) (Table 4.7). Table 4.7 also details the characteristics of the donors, including age, post-mortem autopsy delay and cause of death, and shows that variables such as age and cause of death do not seem to affect the density of [ $^{125}$ I]-GAL binding in the nodose ganglion. In addition, non-specific binding in the presence of excess unlabelled rat GAL was unable to be detected and was not subtracted from total binding (Figure 4.18B).

**FIGURE 4.18**

$[^{125}\text{I}]$ -GAL binding sites in human inferior vagal ganglia.

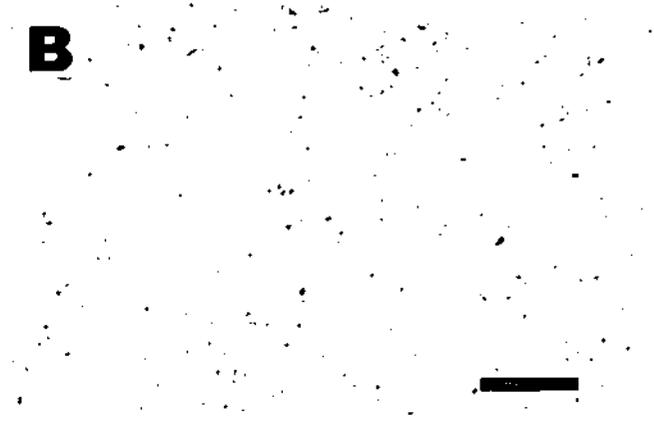
**A-B** – Representative autoradiograms demonstrating the high level of specific binding (A) of  $[^{125}\text{I}]$ -GAL in the human nodose ganglion when compared to the undetectable non-specific binding (B) of  $[^{125}\text{I}]$ -GAL in the presence of unlabeled rat GAL. Scale bar represents 2.25mm.

**C-D** – Dark-field (C) and corresponding light-field (D) photomicrographs of human nodose ganglia sections that were apposed to coverslips previously dipped in photographic emulsion showing the presence of  $[^{125}\text{I}]$ -GAL binding sites. Note the localisation of silver grains, which represent the presence of  $[^{125}\text{I}]$ -GAL binding sites, over large individual cell bodies in panel C. Scale bar represents 170 $\mu\text{m}$ .

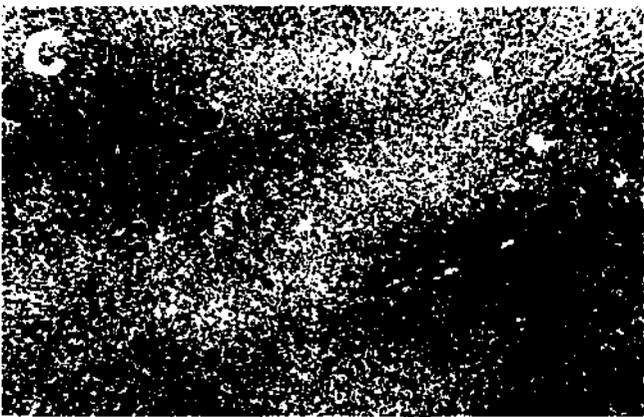
**A**



**B**



**C**



**D**

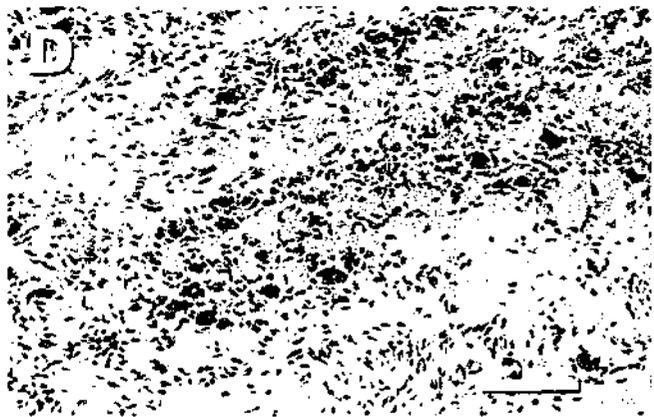


TABLE 4.7

AGE	SEX	DEATH/AUTOPSY DELAY	CAUSE OF DEATH	[ <sup>125</sup> I]-GAL BINDING <sup>1</sup>
18	M	2 DAYS	Asphyxiation by hanging	31±1
62	M	2 DAYS	Severe coronary atherosclerosis	32±1
77	M	2 DAYS	Multiple injuries	26±1
N/K <sup>2</sup>	M	5 DAYS	Ischaemic heart disease	23±1

[<sup>125</sup>I]-GAL binding site density in human nodose ganglia from four donors, including details of donors.

<sup>1</sup>: [<sup>125</sup>I]-GAL binding is expressed as mean DPM/mm<sup>2</sup> ± S.E.M. for 4 sections per subject.

<sup>2</sup>: N/K denotes not known.

To determine whether binding sites were confined to cell bodies or axons, nodose ganglia sections were apposed to coverslips that had been dipped in photographic emulsion and then examined using a microscope. As shown in Figure 4.18C, dense clusters of silver grains were centred over large individual neuronal cell bodies and the smaller satellite cells surrounding them. In slices of the human nodose that were examined, these large neurons had cell bodies with average diameters between 14 and 20µm (Figure 4.18D), and the vast majority (>90%) of these neurons were associated with [<sup>125</sup>I]-GAL binding sites. Figure 4.18C also clearly shows that there is a sparse distribution of silver grains in regions of the human nodose ganglion that are relatively devoid of large neuronal cell bodies.

#### 4.5.4 DISCUSSION

The present study utilised *in vitro* receptor autoradiography to visualise binding sites for GAL within the human nodose ganglion. Light microscopic examination revealed that [<sup>125</sup>I]-GAL binding sites were primarily localised over cell bodies of vagal afferent neurons. Rather diffuse binding of [<sup>125</sup>I]-GAL was also detected on fibres, indicating the possibility of axonal transport of binding sites along the vagus nerve. These results, together with previous reports of

GAL-ir and prepro-GAL mRNA in the nodose ganglia of other species (Philippe *et al.*, 1990; Calingasan & Ritter, 1992), supports the hypothesis that GAL may have a modulatory role in vagal afferent transmission.

These data add to previous reports that have detected receptors for various neurotransmitters, such as NPY, dopamine (D<sub>2</sub>) and ACh (nicotinic), within the human nodose ganglion and vagus nerve (Lawrence *et al.*, 1995; McLean *et al.*, 1997; Ashworth-Preece *et al.*, 1998). Furthermore, binding sites for these neurotransmitters/modulators have also been detected in the rat nodose ganglion or vagus nerve (Moran *et al.*, 1990; Lawrence *et al.*, 1995; Moriarty *et al.*, 1997; Zhou *et al.*, 1997; Ashworth-Preece *et al.*, 1998), demonstrating that there is apparently a substantial degree of homology in the neurochemistry of the nodose ganglion between the rat and the human. Rat and human forms of GAL also share a high degree of homology, with a difference of only four amino acids between the two peptides (Vrontakis *et al.*, 1987; Schmidt *et al.*, 1991). The present study demonstrated that iodinated rat GAL can bind to GAL receptors within the human nodose ganglion, and this binding can be fully displaced by unlabelled rat GAL. This result suggests that the differences in amino acid sequence do not occur in regions of the GAL peptide that are important for receptor binding, thus enabling the use of rat GAL for binding studies in human preparations. Moreover, studies have reported similar biological activity between human and rat GAL in isolated rat fundus muscle strips and in the inhibition of insulin release from a glucose-sensitive rat insulinoma cell line (Schmidt *et al.*, 1991; McKnight *et al.*, 1992).

Immunohistochemical studies have demonstrated the presence of GAL-ir fibres and cell bodies in the NTS in subnuclei where vagal afferents have been shown to terminate (Kalia & Sullivan, 1982), such as the medial subnucleus (Melander *et al.*, 1986b; Härfstrand *et al.*, 1987). Furthermore, the presence of GAL receptors in the NTS (Melander *et al.*, 1988) suggests that these receptors may participate in the regulation of various physiological functions through the modulation of vagal afferent transmission. While there is limited evidence of a functional role at this level for GAL receptors, previous studies have reported that microinjection of GAL into the rat NTS can alter cardiovascular function and feeding behaviour (Chen *et al.*, 1996a; Shih *et al.*, 1996; Koegler & Ritter, 1998). Further studies are therefore required, both in rats and in humans, to determine if GAL can indeed regulate and/or participate in the central dissemination of visceral sensory information transmitted via the vagus nerve. Moreover, these data demonstrate the importance of confirming the presence of neuropeptide receptors in human neural tissue, such that potential therapeutic targets can be identified.

# IMMUNOHISTOCHEMISTRY



## Antibody an'ti-bod"e

Noun - an immunoglobulin molecule that has a specific amino acid sequence by virtue of which it interacts only with the antigen that induced its synthesis.

# CHAPTER 5

## IMMUNOHISTOCHEMISTRY

### 5.1 INTRODUCTION

For over half a century, immunological techniques have been utilised to aid in the identification and distribution of particular peptides, receptors or enzymes in various central and peripheral tissues in a number of species (Melander *et al.*, 1985; Melander *et al.*, 1986b; Yang *et al.*, 1996; Caberlotto *et al.*, 1998). In addition, immunohistochemistry with either light or electron microscopic evaluation has led to the determination of the cellular and subcellular localisation and anatomical distribution of a particular neuropeptide or its markers throughout the CNS and periphery. Immunohistochemistry has therefore proved to be an invaluable experimental tool, particularly because it can be used in conjunction with other histochemical techniques such as retrograde or anterograde tracing to provide information on the phenotype of afferent or efferent projections within the CNS (Zardetto-Smith & Gray, 1990). Moreover, the simultaneous use of a number of different antibodies has provided detailed evidence of the coexistence of particular neuropeptides within the same neuron (Melander *et al.*, 1986c).

This chapter uses immunohistochemistry to detect the presence of Fos protein, the product of the IEG or proto-oncogene, *c-fos*, in the CNS of WKY rats. *C-fos*, together with other members of the fos gene family (*fosB*, *fra1* and *fra2*) and related IEGs (*jun* and zinc finger gene family), are rapidly expressed in response to extracellular stimuli (Melia *et al.*, 1994; Cullinan *et al.*, 1995; Hughes & Dragunow, 1995; Senba & Ueyama, 1997; Stamp & Herbert, 1999). These IEGs encode regulatory proteins, with Fos and Jun formed from translation of the IEGs *c-fos* and *c-jun* respectively, forming a heterodimer called AP-1 that can bind to target genes and regulate their transcription (Senba & Ueyama, 1997).

Increased synthesis of *c-fos* mRNA and Fos protein have been detected in the rat CNS following many stimuli and stressors such as restraint, footshock, hypoxia, hypertonic saline administration, drug-induced hypotension and handling (Sharp *et al.*, 1991; Chen & Herbert,

1995; Cullinan *et al.*, 1995; Li *et al.*, 1996a; Minson *et al.*, 1997; Teppema *et al.*, 1997; McLean *et al.*, 1999). Thus, the presence of *c-fos* mRNA or Fos enables the identification of neurons activated by a particular stimulus, and can provide detailed information regarding the neuronal pathways associated with the central response to that stimulus. Furthermore, an insight into the role of neuropeptides in the central stress response can be gained by combining Fos immunohistochemistry with an additional technique, such as tracing and a microinjection. As the Fos protein is confined to the cell nucleus (Hughes & Dragunow, 1995; Herrera & Robertson, 1996), detection of various neuropeptides in the cytoplasm can demonstrate the phenotype of neurons activated by a particular stimulus. Another approach is the peripheral or central administration of an agonist or antagonist prior to exposure to the stimulus or stress, and changes in *c-fos* mRNA or Fos production provides information regarding the neurochemical modulation of particular neurons involved in the stress response.

It is this latter approach that has been utilised in this chapter. The opioid system will be the focus of the following experiments, particularly in light of the findings of chapters 3 and 4, in addition to the evidence obtained from previous studies outlined previously. Central (i.c.v.) administration of naloxone and naltrexone, non-selective opioid receptor antagonists, have been shown to decrease feeding, increase plasma luteinising hormone concentration, modulate nociception and increase the number of Fos positive cells in the Ce, BNST and NAcc (Yu *et al.*, 1991; Murase *et al.*, 1996; Chiba *et al.*, 1998; Carr *et al.*, 1999). Peripheral (i.v. or s.c.) administration of naloxone or naltrexone has produced significant alterations in Fos immunohistochemistry in central regions such as the AP, NTS, RVLM, supramammillary nucleus, BNST and Ce (Carr *et al.*, 1998; Gestreau *et al.*, 2000). In the study by Gestreau and colleagues, i.v. administration of naloxone methiodide, a non-selective opioid antagonist that does not cross the blood-brain barrier, did not produce the same changes in Fos production as those produced by i.v. naloxone, suggesting that naloxone is having its effects within the CNS (Gestreau *et al.*, 2000).

Central administration of naloxone or opioid agonists in rats exposed to various stressors have produced changes in the function of a number of physiological systems. Yamauchi and colleagues demonstrated that i.c.v. administration of an anti- $\beta$ -endorphin rabbit  $\gamma$ -globulin suppressed the restraint-induced elevation of ACTH release in conscious rats (Yamauchi *et al.*, 1997). Studies of the effects of opioid agonists on the sympathoadrenal system during stress have produced contrasting results.  $\beta$ -Endorphin, DAME, morphiceptin and DADLE suppressed the immobilisation-induced elevation in blood adrenaline concentrations when administered

centrally (i.c.v.) (Nakamura *et al.*, 1989), while i.c.v. DAGO potentiated the plasma NAdr and adrenaline increases observed in conscious rats exposed to restraint (Marson *et al.*, 1989). In terms of the cardiovascular system, pretreatment of rats with DAGO (i.c.v.) suppressed the restraint-induced tachycardia and hypertension, while i.c.v. naloxone attenuated the hypertension elicited by social isolation and an air-jet stress paradigm (Kapusta *et al.*, 1989; Marson *et al.*, 1989; Jiménez *et al.*, 1990). However, intra-PVN administration of naloxone in conscious, restrained rats had no effect on plasma NAdr, HR or MAP, while it potentiated the restraint-induced rise in plasma adrenaline levels (Kiritsy-Roy *et al.*, 1986). Thus, studies such as these demonstrate that the central opioid system is involved in the modulation of various components of the response to psychological stressors; however, the brain regions and pathways associated with this modulation are unclear. Therefore, this chapter aims to investigate the opioid-mediated modulation of the central response to acute restraint, a psychological stressor used in chapters 3 and 4. Naloxone will be administered centrally (i.c.v.) to inhibit the activity of the central opioid system in the normotensive WKY rat. Fos immunohistochemistry, in particular the number of Fos-positive cells, will be quantified and compared in selected regions of the CNS of naloxone- and saline-treated rats, in nuclei such as the PVN, SON, PAG, LC, VLM and NTS. Previous studies have shown that not only are these regions sensitive to activation by restraint, they are also involved in various effector pathways of the neural stress response (Boone & McMillen, 1994a; Chen & Herbert, 1995; Cullinan *et al.*, 1995). Moreover, these regions also contain opioids and/or are innervated by opioid-containing neurons (see section 1.3.1). Thus, any significant changes in Fos production induced by naloxone in these regions may suggest that the central opioid system has an influence on the neuroendocrine, cardiovascular or behavioural response to restraint stress.

## 5.2 METHODS

### 5.2.1 INTRACEREBROVENTRICULAR (I.C.V.) DRUG ADMINISTRATION

#### 5.2.1.1 *Implantation of guide cannulae*

Male WKY rats (360-405g) were anaesthetised with sodium methohexitone (60mg/kg; i.p.). A heating mat and cotton wool were used to keep the rat warm, and anaesthesia was maintained throughout the surgery using sodium methohexitone as required (10-15mg/kg; i.p.). The depth of anaesthesia was monitored closely throughout the surgical procedure, and the absence of a withdrawal response following a paw pinch indicated an adequate level of anaesthesia required for surgery. The corneal reflex was also checked on a regular basis to monitor the level of anaesthesia.

The rat was placed in a stereotaxic head frame (David Kopf instruments, USA), with the incisor bar set at -3.3mm below the interaural point. After an injection of the local anaesthetic lignocaine (0.5ml of 1% solution; s.c.; Delta West) that was directed at the skin overlying the skull, a midline incision was made at the top of the skull and the skin was reflected to expose the skull and bregma. A 3mm burr hole was drilled (Dremel moto-tool Model 396, Racine, WI, USA) in the occipital bone above the lateral ventricle at the following coordinates derived from (Paxinos & Watson, 1986) (A-P : -0.7mm, M-L : -1.4mm from bregma).

A custom made stainless steel guide cannula (external diameter - 0.025"; Small Parts Inc., USA) (Figure 5.1A) was then slowly inserted into the burr hole and stereotaxically lowered to a depth of 2.7mm below the skull surface (Figure 5.1B). To maintain the guide cannula in this position, dental cement (Pulver and Flussigkeit; Paladur, Kuler and Co., Germany) was applied to the surface of the skull and cannula with additional support provided by three screws (1.4mm x 3mm; Mr Specs, Mordialloc, Vic., Aust) inserted in the skull surrounding the guide cannula. Once the dental cement had dried and set, the incision was closed with standard sutures. Buprenorphine (0.01mg in 0.1ml; i.m.; Sigma Chemical Co., Australia) was administered immediately after surgery for post-operative analgesia and Cicatrin antibiotic powder (Wellcome Ltd, Australia) was applied to the surgical area. The rat was then placed under a warm lamp until the effects of the anaesthetic had diminished. All rats were given at least 3 days to recover

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**FIGURE 5.1**

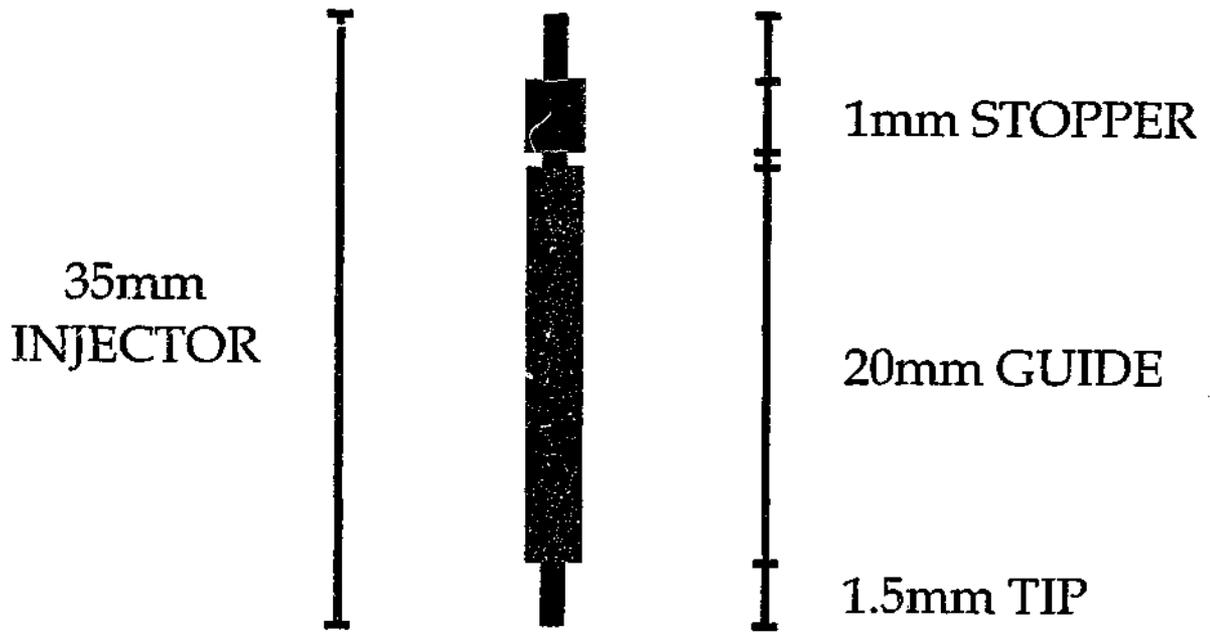
A - Schematic diagram of the custom-made injector and guide cannula. The 20mm guide cannula is chronically fixed to the rat skull, such that the 1.5mm tip of the injector projects into the lateral ventricle when the injector is inserted into the guide cannula.

B - Schematic representation of the guide cannula and injector used for i.c.v. drug administration following implantation in the rat brain at the A-P coordinates of  $-0.7\text{mm}$  and the M-L and D-V coordinates as shown.

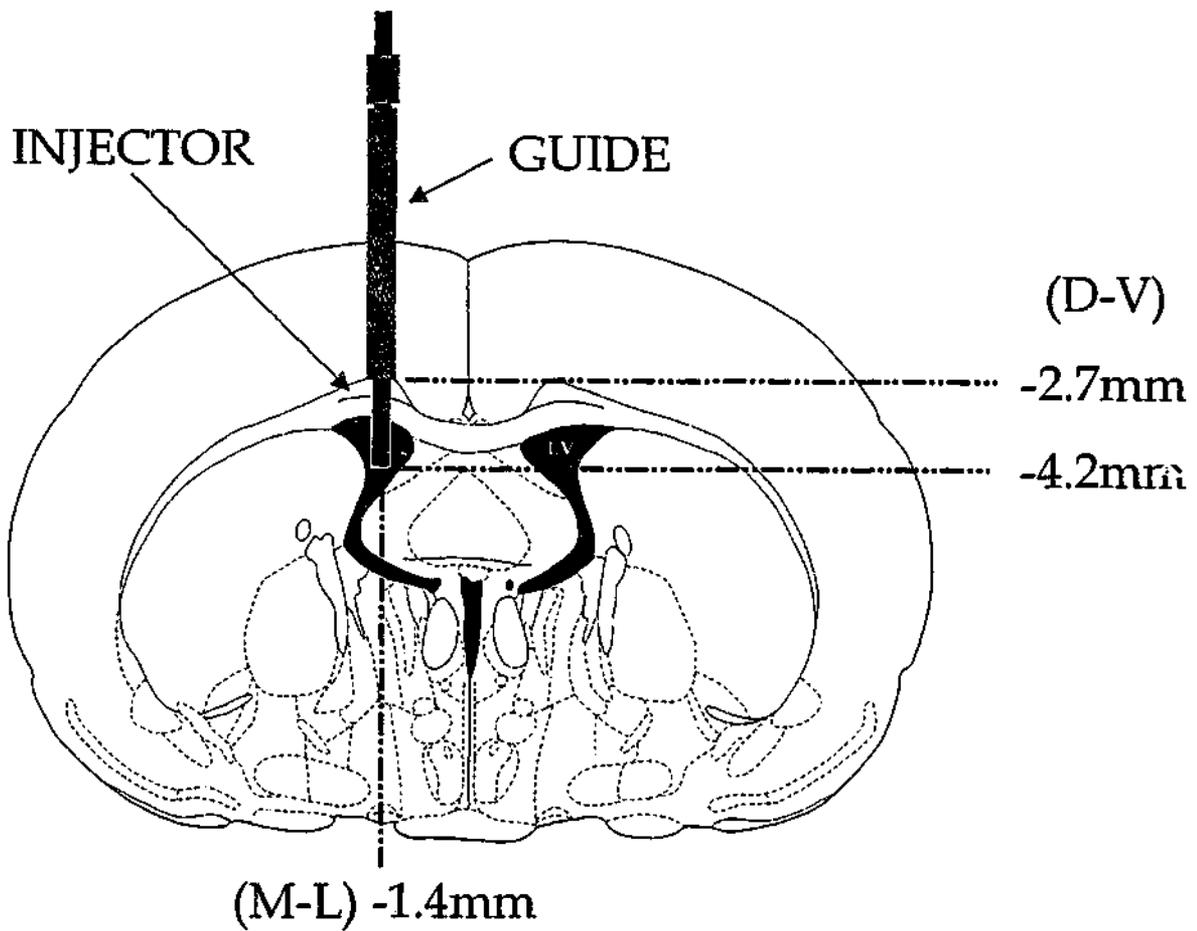
For abbreviations, see page xvii.

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**A**



**B**



from surgery in their home cages with food and water *ad libitum* before they were subjected to any further procedures.

### 5.2.1.2 Confirmation of guide cannulae position

Two days before experimentation (i.e. 5 days after surgery), the position of the guide cannula within the lateral ventricle was confirmed using the following method. Angiotensin II (20 $\mu$ M; 5 $\mu$ l; American Peptide Co.) was injected i.c.v. (see below) and a rapid drinking response (<45 seconds) indicated that the injector was placed within the lateral ventricle. Further confirmation was obtained from histological examination of the injection site at the completion of the experiment.

### 5.2.1.3 Drug administration

The following injection procedure was used for all i.c.v. drug administration. The injector was a 35mm segment of stainless steel tubing (external diameter – 0.012"; Small Parts Inc., USA) with a stainless steel collar secured 21.5mm above the base of the injector to maintain an injection depth of 1.5mm beyond the tip of the guide cannula (see Figure 5.1A). The injector was attached to a 25 $\mu$ l Hamilton syringe (SGE, Australia) by a length of SP45/SP10 polyethylene tubing (Datamasters, Australia) that had been filled with the appropriate solution. The length of the tubing connecting the syringe and injector was sufficiently long to allow remote injection of the rats while they remained in their home cage. The injector was subsequently lowered into the lateral ventricle via the guide cannula and a 5 $\mu$ l drug volume was administered using the Hamilton syringe, with movement of an air bubble along the SP45 polyethylene tubing used to monitor drug delivery. The injector was removed from the guide cannula 1-2 min after completion of the injection. Throughout the entire injection procedure, the rat remained in its home cage and was not handled to minimise the potential effects of this minor stress.

## 5.2.2 PRELIMINARY STUDY

A preliminary study was undertaken to select the appropriate dose of naloxone (Sigma Chemical Co., Australia) for use in subsequent experiments. While literature reports detailed a wide range of effective i.c.v. doses of naloxone (<1 nmol to >250 nmol) (Miynarska, 1990; Murase *et al.*, 1996), it was decided that the selected dose of naloxone should have a minimal effect on BP. Furthermore, despite the availability of a number of alternative indicators of effective opioid blockade (e.g. pain threshold, body temperature) that were available for use, I chose BP as many of the brain regions of interest are sensitive to changes in BP. Thus, any changes in Fos production observed in these regions could be attributed to the stress rather than changes in BP induced by naloxone.

### 5.2.2.1 Tail artery cannulation

Male WKY rats (360-395g; n=4) were implanted with guide cannulae directed at the lateral ventricle for drug administration (see section 5.2.1.1). Six days after this surgery, the rats were anaesthetised with sodium methohexitone (60mg/kg; i.p.) and anaesthesia was maintained as outlined above. An anterior 1-1.5cm incision was made on the ventral side of the tail and the tail artery was exposed by carefully separating the surrounding tissue with forceps. A previously prepared arterial catheter (5-7cm segment of SP10 polyethylene tubing inserted and fixed to SP45 polyethylene tubing) filled with saline (0.9%; Delta West) and heparin (0.4%; David Bull Laboratories) was then inserted into the abdominal aorta via the caudal tail artery in an anterior direction and secured in place. The arterial line was then tunnelled subcutaneously using a trocar rod and exteriorised and anchored at the posterior neck area between the scapulae. Incisions that were made in the tail and neck were subsequently closed with standard sutures. All rats were returned to their home cage and given 24 hours to recover from surgery before experimentation, during which time they had free access to food and water.

On the following day, naloxone (100, 250 or 350 nmol; each in a 5 $\mu$ l injection volume) and saline (5 $\mu$ l) were administered i.c.v. and the effects on MAP were monitored using a pressure transducer (Gould P23ID, USA) attached to a Grass polygraph (79D, Grass Instruments, USA). At least 30 min separated each injection.

## 5.2.3 EFFECTS OF NALOXONE AND SALINE ON FOS PRODUCTION

### 5.2.3.1 *Injection procedure*

Male WKY rats (365-405g) were implanted with guide cannulae targetted at the lateral ventricle as detailed in section 5.2.1 and allowed to recover for at least 7 days. On the day before the experiment, rats were transferred to the experimental room to acclimatise to the new surroundings. Furthermore, noise was minimal in this room to reduce any variation caused by unnecessary environmental stress. Between 10:00h and 12:00h on the day of the experiment, rats received i.c.v. microinjection of either naloxone (100nmol; n=3) or saline (control; n=3). Rats were processed simultaneously in pairs, with one rat receiving i.c.v. naloxone and the other receiving i.c.v. saline.

Following the i.c.v. injection of naloxone or saline, rats remained in their home cages for 2 hours, were deeply anaesthetised with sodium pentobarbitone (80 mg/kg; i.p.; Boehringer Mannheim, Germany), perfused and subsequently processed for Fos immunohistochemistry as described in section 5.2.6.

## 5.2.4 RESTRAINT AND FOS PRODUCTION

A time course analysis was completed to determine the optimal time delay between conclusion of the restraint stimulus and perfusion of the rats to produce a maximal and reproducible expression of the Fos protein in the CNS. Male WKY rats (335-385g) were transferred to the experimental room on the day prior to the experiment and subjected to the 60 min restraint protocol described in section 2.3 between 10:00h and 12:00h. NOTE – these rats did not have any implanted i.c.v. cannulae. At the conclusion of the restraint session, the rats were removed from the restraint tube and remained in their home cages for a further 30 min (n=2), 60 min (n=2) or 120 min (n=2). At the end of this time, rats were deeply anaesthetised with sodium pentobarbitone (80 mg/kg; i.p.), perfused and subsequently processed for Fos immunohistochemistry as described in section 5.2.6.

## 5.2.5 NALOXONE (I.C.V.) AND RESTRAINT-INDUCED FOS PRODUCTION

Male WKY rats (365-405g) were implanted with i.c.v. guide cannulae as described in section 5.2.1 and allowed to recover for at least 7 days. Rats were then injected i.c.v. with either naloxone (100nmol; n=4) or saline (n=4) as described earlier, with rats again processed simultaneously as a pair, with one rat receiving an i.c.v. injection of naloxone and the other receiving i.c.v. saline (section 5.2.3.1). Immediately following injection of either naloxone or saline, all rats were subjected to one session of restraint stress in their home cages for 60 min as previously described (section 2.3). During the restraint session, general behaviour was observed and recorded. At the completion of the restraint session, rats remained in their home cages for 60 min (see section 5.3.3.1), after which they were deeply anaesthetised with sodium pentobarbitone (80mg/kg; i.p.) and perfused as described below (section 5.2.6).

## 5.2.6 FOS IMMUNOHISTOCHEMISTRY

### 5.2.6.1 *Transcardial perfusion and collection of brain sections*

Rats were transcardially perfused with 100ml of 0.1M PBS (pH 7.4; in the following sections, PBS refers to 0.1M PBS with pH 7.4) and 400ml of 4% PFA via the ascending aorta (Masterflex pump, Cole-Parmer Instrument Co., Chicago, USA). Once the perfusion had finished, the needle was removed from the heart, the brain was dissected and separated into the forebrain and pons/medulla. Both sections of the brain were cryoprotected overnight in PBS containing 20% sucrose at 4°C.

The following day, the brains were removed from the sucrose solution and mounted on a chuck with Tissue-Tek® O.C.T. Using a freezing microtome (Leitz, Germany), 50µm coronal sections were collected from the forebrain at the level of the hypothalamus and amygdala, pons/midbrain at the level of the PAG/LC and medulla oblongata at the level of the VLM/NTS. Brain sections were placed in PBS in 48 well plates (11.3mm diameter) for further immunohistochemical processing.

### 5.2.6.2 Reference maps

A reference map for assistance in the identification of specific brain regions was collected for each rat. For every 2 brain sections collected for Fos immunohistochemical processing at the level of the forebrain and pons/midbrain described above, a further section was included in the reference set. In the medulla oblongata, one section was included in the reference set for every 3 sections collected for subsequent processing for Fos protein. The brain sections contained in each reference set were mounted onto microscope slides using 0.5% gelatin and left to dry overnight. On the following day, tissue sections were stained in 0.1% thionin, dehydrated in ethanol (70, 90 and 3 X 100%; 30 sec each), cleared in HistoClear and coverslipped using DPX mountant.

### 5.2.6.3 Immunohistochemistry

The following immunohistochemistry procedure has been previously published and used extensively in our laboratory (Lawrence *et al.*, 1998; McLean *et al.*, 1999). Brain sections were transferred to a preblock solution containing 10% normal rabbit serum (NRS) and 0.3% Triton X-100 in PBS and incubated for 15 min to reduce background staining. Following the preblock, sections were washed in PBS (3 X 5 min). Sections were then transferred to the primary antibody mix containing 2% NRS, 0.3% Triton X-100 and the sheep polyclonal anti-Fos antisera (1/3000; Genosys, England) in PBS and incubated overnight at 4°C.

The next day, sections were subjected to 3 X 10 min washes in PBS at room temperature and incubated in a secondary antibody solution containing 2% NRS, 0.3% Triton X-100 and rabbit anti-sheep biotinylated secondary antibody (1/500; Silenus, Australia) in PBS for 60 min at room temperature. Sections were washed again in PBS (3 X 10 min). Subsequently, tissue sections were incubated for 60 min at room temperature in streptavidin-horseradish peroxidase (strep-HRP; 1/500; Silenus, Australia) containing 2% NRS and 0.3% Triton X-100 in PBS. Following the strep-HRP incubation, sections were washed in PBS (3 X 10 min).

The following procedure was adapted from Llewellyn-Smith (Llewellyn-Smith *et al.*, 1992). To visualise the presence of Fos-ir, 3',3'-diaminobenzidine (DAB) (Sigma Chemical Co., Australia) was used as a chromagen in the presence of the enzyme glucose oxidase (Sigma Chemical Co., Australia) to generate a black reaction product. After the sections had been

washed, they were incubated in a solution containing 0.5mg/ml DAB, 0.004%  $\text{NH}_4\text{Cl}$ , 0.2% D-glucose and 0.04% nickel ammonium sulphate in PBS for 10 min. The enzyme glucose oxidase (1 $\mu\text{l}/\text{ml}$ ; Sigma Chemical Co., Australia) was then added and mixed. Sections remained in this solution until the black amorphous reaction product could be visualised with the naked eye. To terminate the colour reaction, sections were washed in PBS containing 50mM Tris (pH 7.4; 3 X 10 min).

#### *5.2.6.4 Mounting of sections and histology*

Sections were mounted onto microscope slides using 0.5% gelatin and left to dry overnight. On the following day, tissue sections were dehydrated in ethanol (70, 90 and 3 X 100%; 30 sec each), cleared in HistoClear® and coverslipped using DPX® mountant.

#### *5.2.6.5 Assessment of Fos-ir and specificity of Fos staining*

A light microscope was used to visualise cells containing Fos-ir, and any cells containing black nuclear staining that was distinct from background were counted as Fos-positive. These cells were quantified in all focal planes in each section. All regions were identified with the assistance of a reference set of adjacent brain sections and a rat atlas (Paxinos & Watson, 1986). Photographs were taken as described in section 2.11.

When sections were processed using the immunohistochemistry protocol in the absence of either the primary antibody or secondary antibody, Fos-positive nuclei were not detected, demonstrating that the Fos staining in these experiments was specific.

### **5.2.7 DATA ANALYSIS**

The effects of saline and various doses of naloxone on MAP were quantified at 1, 5 and 10 min after injection. Five measures of systolic and diastolic BP were obtained from the trace, averaged and used to calculate MAP for each time point using the formula described earlier

(section 2.4.2). Data were then compared using a two way repeated measures ANOVA followed by a post-hoc Bonferroni test.

When Fos-positive cells were quantified in bilateral nuclei, data were obtained from both sides of the section and represented as a sum of both left and right. Data were expressed in a number of ways. To demonstrate the effects of i.c.v. naloxone and saline administration on Fos expression in unstressed or stressed rats, the amount of Fos-positive cells were quantified and expressed as the total number for an entire region. Statistical comparison of these results was achieved using an unpaired student's *t*-test. To provide a representation of the distribution of cells containing Fos-ir over a rostro-caudal axis within a specific region, Fos-positive cells were quantified in either 2 (forebrain and pontine sections) or 3 (medulla oblongata sections), summed and plotted on a graph. This method permitted an accurate anterior-posterior reconstruction of a nucleus, and the fact that sections were also collected for a reference set was accounted for when assigning anatomical levels. A two way repeated measures ANOVA followed by a post-hoc Bonferroni test was used to statistically compare the rostro-caudal pattern of Fos-positive cell distribution between saline- and naloxone-treated rats exposed to restraint. A one way ANOVA with post-hoc Student Newman-Keuls test was employed to compare the number of Fos-positive cells contained in three subregions of the NTS – the caudal (caudal of AP), subpostremal and rostral (rostral of AP). In all cases, a level of  $P < 0.05$  was considered to be statistically significant.

## 5.2.8 MATERIALS AND ABBREVIATIONS

For all materials used in this chapter, refer to sections 2.14.1 and 2.14.4. For all abbreviations, refer to page xvii.

## 5.3 RESULTS

### 5.3.1 PRELIMINARY STUDY

Naloxone was injected centrally (i.c.v.; 100, 250 and 350 nmol/5 $\mu$ l) in conscious rats and subsequent effects on MAP were observed and recorded continuously for a number of hours. To determine the BP effects of these i.c.v. injections of saline or naloxone, MAP was quantified at 1, 5 and 10 min after the injection. Variable transient changes in MAP were observed immediately after the i.c.v. administration of naloxone. As shown in Figure 5.2, the lowest dose of naloxone used in this preliminary study (100nmol) had no significant effects on MAP (Figure 5.3). The next dose of naloxone (250nmol) induced a significant rise in MAP of ~8mmHg at both 5 and 10 min post-injection ( $P < 0.05$ ; two way ANOVA). Central (i.c.v.) administration of the highest dose of naloxone (350nmol) also resulted in a significant 8 mmHg MAP increase after 5 min, with a further significant increase of 12mmHg observed 10min after microinjection of this naloxone concentration. Saline microinjections (i.c.v.) did not result in any MAP changes above 1mmHg. Thus, a dose of 100nmol naloxone was used in the following immunohistochemistry experiments.

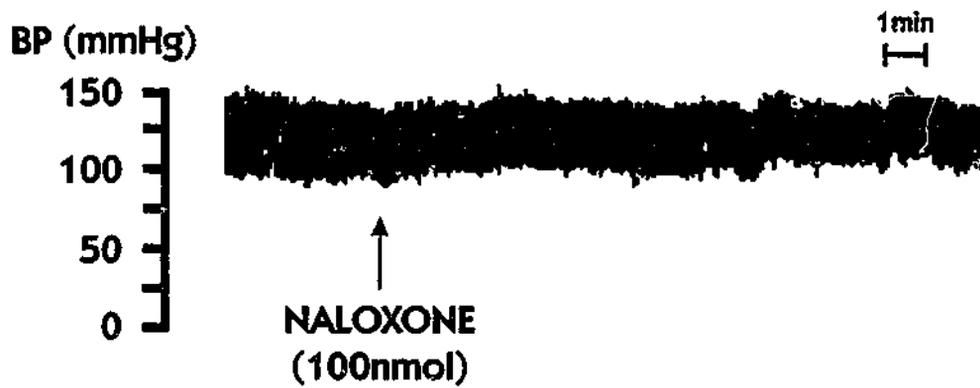
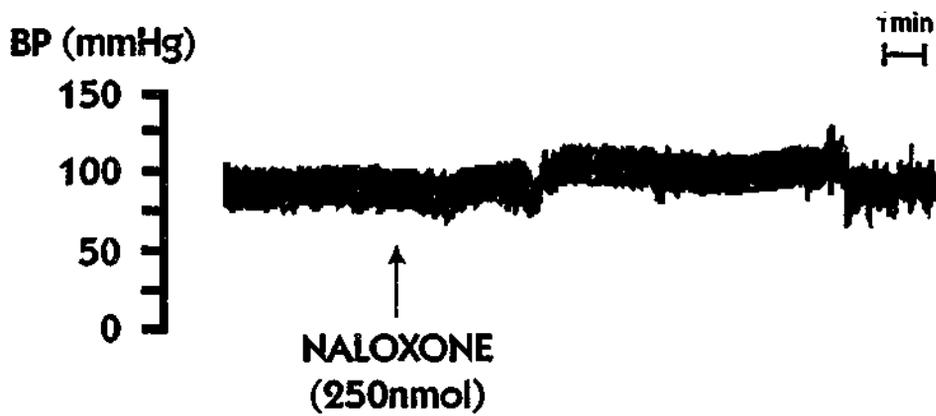
### 5.3.2 I.C.V. NALOXONE AND FOS PRODUCTION

Two hours after i.c.v. administration of the opioid antagonist naloxone (100nmol) or saline, Fos-positive cells were visualised and quantified throughout different levels of the rat CNS in regions such as the Ce, Me, PVN, SON, LC and NTS. In all regions examined, there was no significant effect of i.c.v. naloxone on the number of Fos-positive cells when compared to the saline-treated rats ( $P > 0.05$ ; student's unpaired *t*-test). However, there was a trend towards an increased expression of Fos protein in neurons of the Ce and all regions of the NTS (Table 5.1).

The Ce contains an extremely high basal level of Fos production in rats from both treatment groups, possibly due to a high background expression of Fos. It is not the procedure and any associated stress that has caused this high Fos level in the Ce, as indicated by a low level of Fos production in the Me that is an order of magnitude lower than the Ce. Division of the NTS into 3 portions, the caudal NTS (caudal of AP), subpostremal NTS and rostral NTS (rostral of AP),

**FIGURE 5.2**

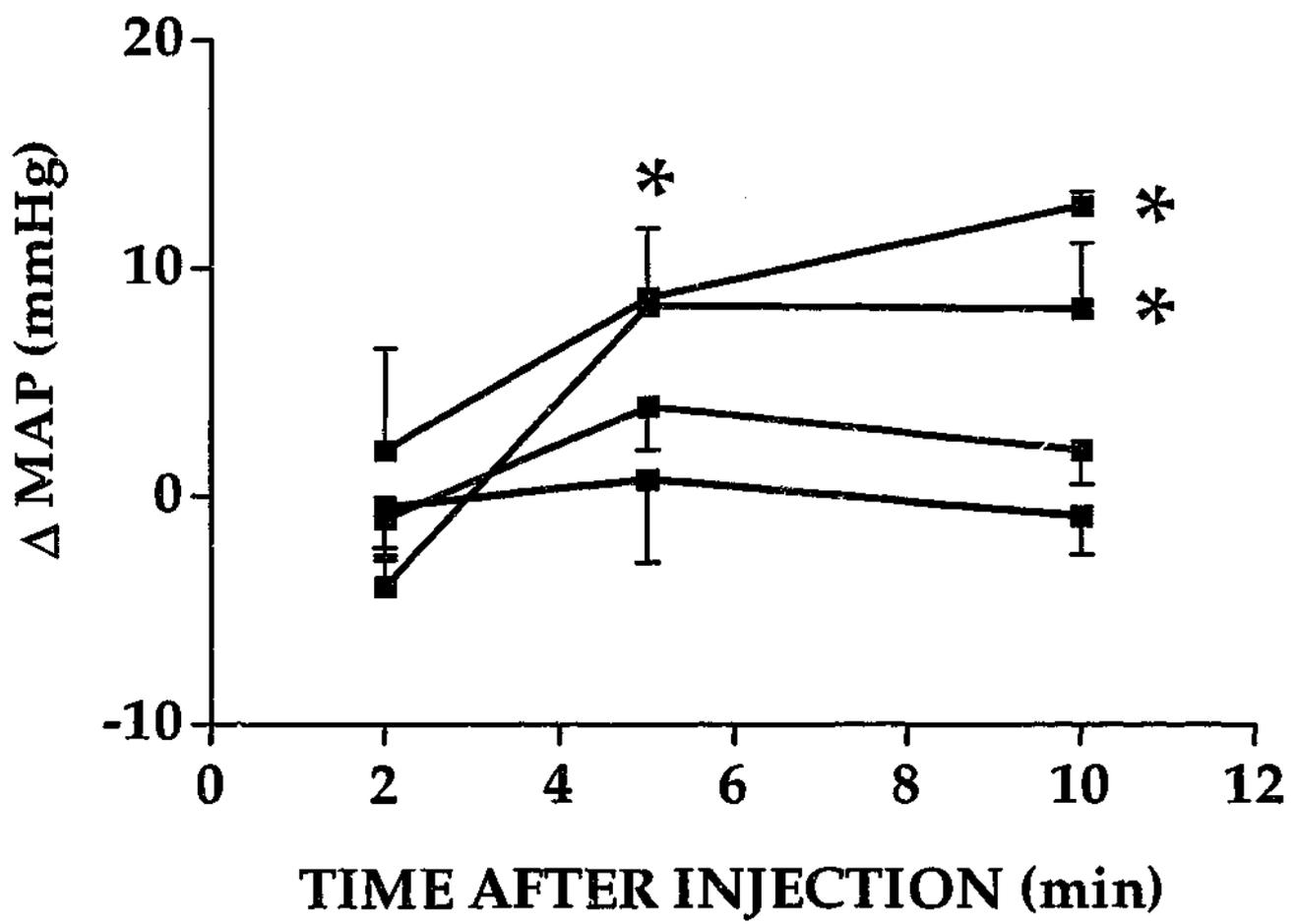
Representative blood pressure (BP) traces demonstrating the effects of i.c.v. microinjection of 5 $\mu$ l of A – saline; B – naloxone (100nmol) and C – naloxone (250nmol) on BP (mmHg) in conscious WKY rats over a 15 min period. An intra-arterial (i.a.) catheter had been implanted in the tail artery of the rat, and BP was monitored via the i.a. catheter that was attached to a pressure transducer and Grass polygraph. Panel A clearly shows that i.c.v. saline has no effect on resting BP, while panel B also demonstrates the lack of BP changes following i.c.v. naloxone (100nmol). A pressor response was observed following i.c.v. administration of 250nmol naloxone (panel C). Scale bar represents 1 min.

**A****B****C**

**FIGURE 5.3**

The effects of i.c.v. microinjection of 5 $\mu$ l of saline (black), naloxone (100nmol; red); naloxone (250nmol; blue) and naloxone (350nmol; green) on mean arterial pressure (MAP) in conscious WKY (n=4). Data are expressed as change ( $\Delta$ ) in MAP (mmHg) at 2, 5 and 10 min post-injection.

\*:  $P < 0.05$ ; two way ANOVA with post-hoc Bonferroni test, with the blue and green stars referring to 250nmol and 350nmol doses of naloxone respectively.



revealed that the subpostremal NTS contained the highest number of Fos-positive cells. However, comparison of the quantity of cells containing Fos-ir in each division of the NTS between rats receiving i.c.v. naloxone and saline did not find any significant differences ( $P > 0.05$ ; student's unpaired  $t$ -test).

**TABLE 5.1**

REGION	SALINE (n=3)	NALOXONE (n=3)
Ce	2869 ± 615	3162 ± 592
Me	186 ± 42	163 ± 64
RVLM	5 ± 4	7 ± 6
NTS	117 ± 68	161 ± 135
Caudal NTS	3 ± 2	5 ± 4
Subpostremal NTS	80 ± 46	102 ± 94
Rostral NTS	34 ± 23	53 ± 36

Effects of i.c.v. saline (control) and naloxone (100nmol in 5 $\mu$ l) on the production of Fos protein in selected regions of the CNS of unstressed rats (n=3 per treatment). Data represent the total number of Fos-positive cells per region and are expressed as mean ± S.E.M.

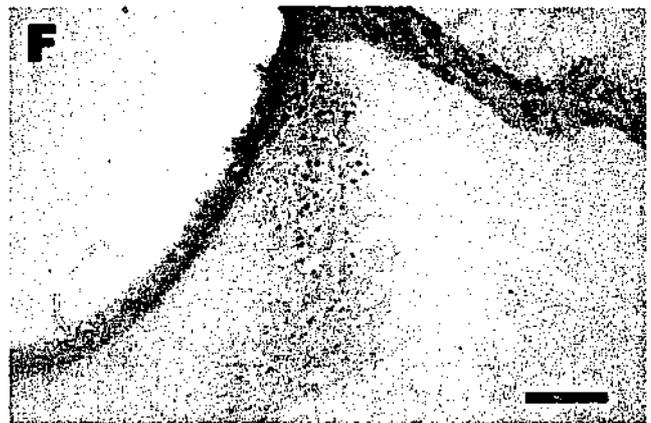
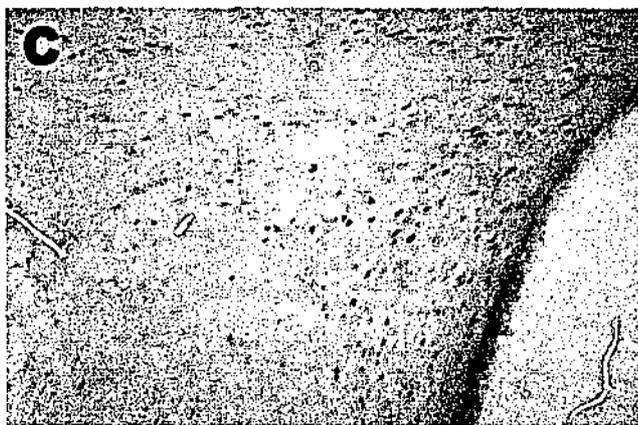
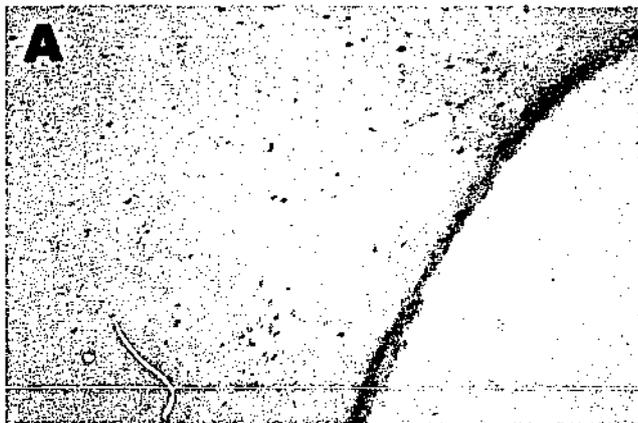
### 5.3.3 RESTRAINT STRESS AND FOS PRODUCTION

#### 5.3.3.1 *Optimisation of protocol*

Preliminary experiments were conducted to determine the appropriate time between the completion of the 60 min restraint period and perfusion to maximise Fos production. A comparison of 30 min, 60 min and 120 min post-restraint periods revealed that the highest and most reproducible levels of Fos production were obtained using a 60 min delay between

**FIGURE 5.4**

Photomicrographs demonstrating the level of expression of Fos-ir in the paraventricular nucleus of the hypothalamus (PVN; A, C, E) and locus coeruleus (LC; B, D, F) in WKY rats exposed to a single 60 min restraint period. At the conclusion of the restraint session, rats were released from the tube and left in their home cage for 30 min (A, B), 60 min (C, D) or 120 min (E, F) before they were deeply anaesthetised (sodium pentobarbitone; 80mg/kg; i.p.) and perfused. The level of expression of Fos protein in the CNS was compared using each of these time periods to determine the optimal experimental conditions for visualisation and quantification of Fos-positive cells. In the PVN, the lowest levels of Fos-positive cells were observed 30 min after cessation of restraint (A), while in the LC, the lowest expression of Fos protein was observed 120 min post-restraint (F). Thus, a 60 min delay between restraint and perfusion was selected for use in subsequent experiments. Scale bar represents 150  $\mu\text{m}$  (A-F).



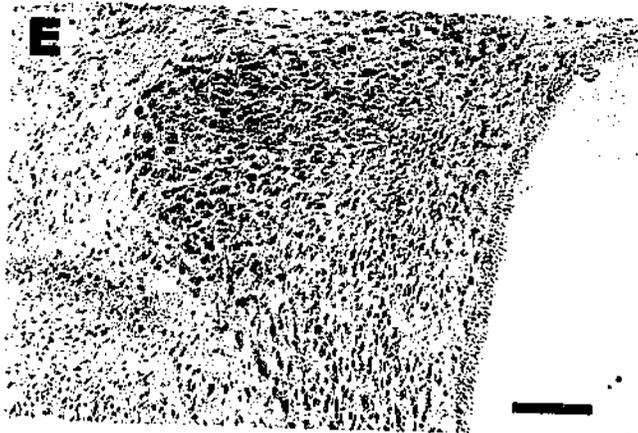
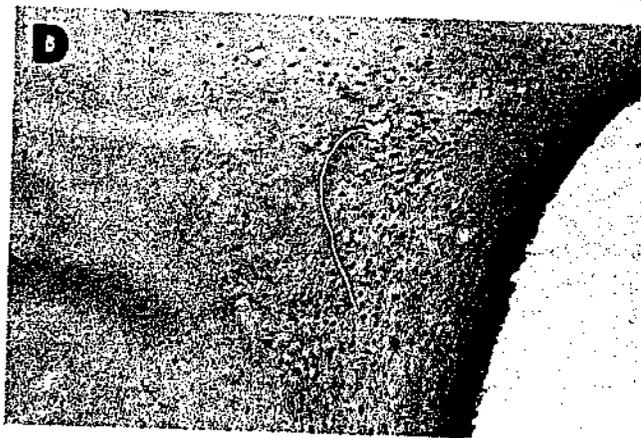
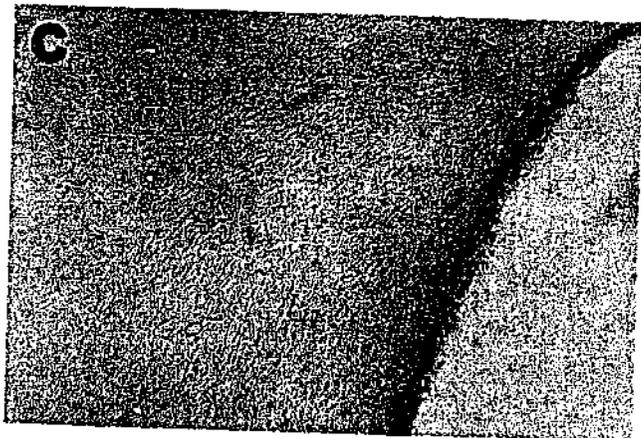
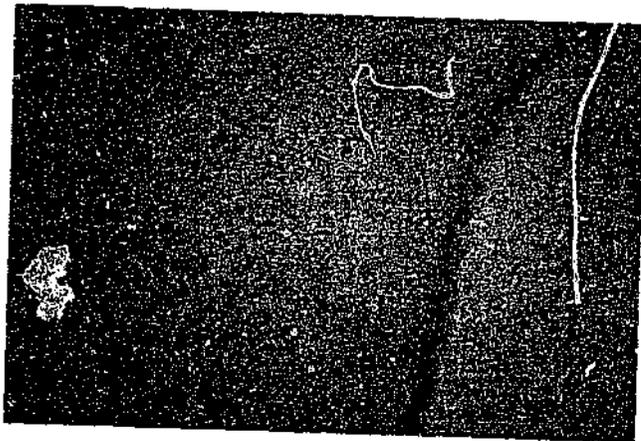
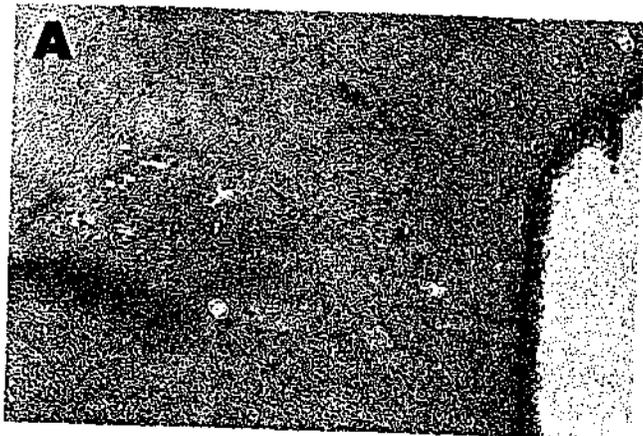
cessation of the restraint stimulus and perfusion. Each experiment, irrespective of the time delay between restraint and perfusion, induced detectable levels of Fos-ir observed in large neuronal populations previously reported to be activated by restraint stress (Chen & Herbert, 1995; Cullinan *et al.*, 1995). Figure 5.4 contains representative sections of the PVN and LC using a post-stress delay period of 30, 60 and 120min. In the PVN, there were detectable levels of Fos production at each of the chosen time points, with the lowest levels of Fos-positive cells observed using a 30 min delay between restraint and perfusion (Figure 5.4A). However, in the LC, the highest levels of Fos production were observed at 30 and 60 min after restraint, with a substantial decrease in the number of Fos-positive cells 120 min after the completion of the restraint paradigm (Figure 5.4F). Therefore, as it appears that similar levels of Fos production were achieved 60 and 120 min after restraint stress in the PVN, the 60 min post-restraint period was selected for future experiments.

#### 5.3.3.2 General observations

Rats were exposed to one session of restraint stress for a period of 60 min. During this restraint period, all rats demonstrated characteristic behavioural responses, including piloerection, vocalisation, defecation/urination and a general state of alertness. Following the 60 min delay between cessation of restraint and perfusion, rats were processed for Fos immunohistochemistry to determine the extent of central activation following exposure to this psychological stressor. When compared to rats that had not been subjected to restraint, acute exposure to restraint induced a significant elevation in Fos protein levels in hypothalamic regions such as the PVN (Figure 5.5), SON (Figure 5.6), Pe, ARC and LHv, amygdaloid regions such as the Me (Figure 5.7) and Ce (Figure 5.8), PVA, Pir (Figure 5.11 and B), LC (Figure 5.9A – D), Teg, PAG (Figure 5.9E – H) and NTS (Figure 5.10 and 5.11). Data obtained from brain regions where Fos-positive cells were quantified are represented in Figure 5.12, and include discrete central nuclei such as the PVN, SON, Me, LC and NTS. Statistical comparison of the levels of Fos production in unstressed and restrained rats receiving i.c.v. saline revealed that restraint increased the number of Fos-positive cells in the majority of regions when compared to unrestrained rats, including the PVN, SON, Me, LC and RVLM ( $P < 0.05$ ; unpaired student's *t*-test). Furthermore, some of the regions where Fos-positive cells were quantified demonstrated a specific rostro-caudal pattern of Fos staining following acute restraint, including the PVN, SON, Me, LC and NTS (Figure 5.13). In the hypothalamic PVN and SON, Fos production was more

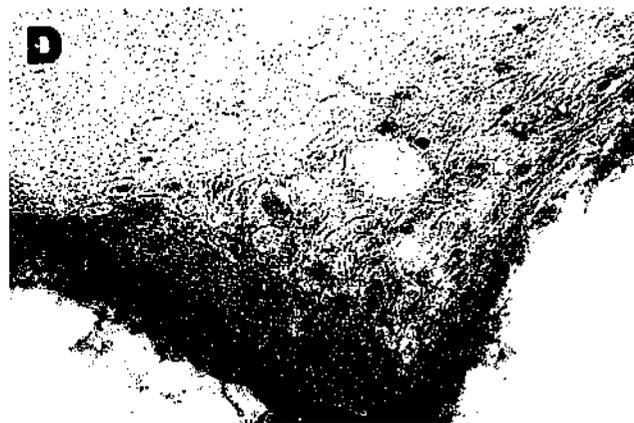
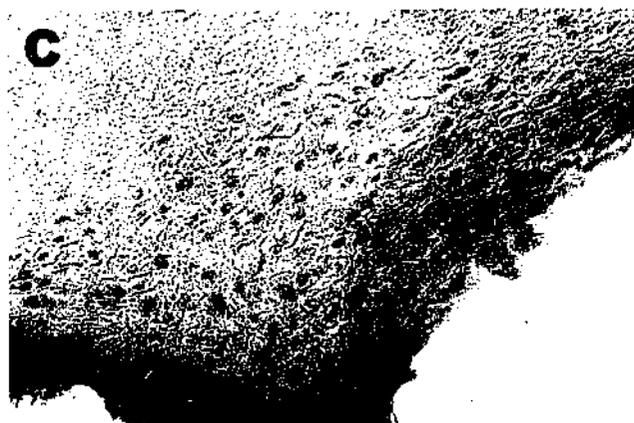
**FIGURE 5.5**

Representative photomicrographs of Fos protein expression in neurons of the paraventricular nucleus of the hypothalamus (PVN) of WKY rats that were microinjected (i.c.v.) with 5 $\mu$ l of saline (n=3; A) or naloxone (100nmol; n=3; B) or WKY that were subjected to a 60 min restraint session that was preceded by an i.c.v. microinjection of saline (n=4; C) or 100nmol naloxone (n=4; D). Panels A and B demonstrate that a similar level of Fos expression was observed in the PVN of unrestrained control rats receiving i.c.v. saline (A) or naloxone (B). A higher number of Fos-positive cells were evident in the PVN of rats exposed to restraint (panels C and D) when compared to the unstressed controls depicted in panels A and B. Panel D demonstrates the elevated expression of Fos protein that was observed in the PVN of rats that had been pretreated with 100nmol naloxone (i.c.v.) immediately preceding the restraint session when compared to restrained rats receiving i.c.v. saline (C). Furthermore, panels C and D also clearly show that the expression of Fos protein induced by restraint was primarily confined to the pPVN, with minimal Fos-ir evident in the magnocellular subregion of the PVN. Panel E represents an adjacent section of the PVN that was thionin-stained for use as an anatomical reference, while the scale bar represents 150 $\mu$ m.



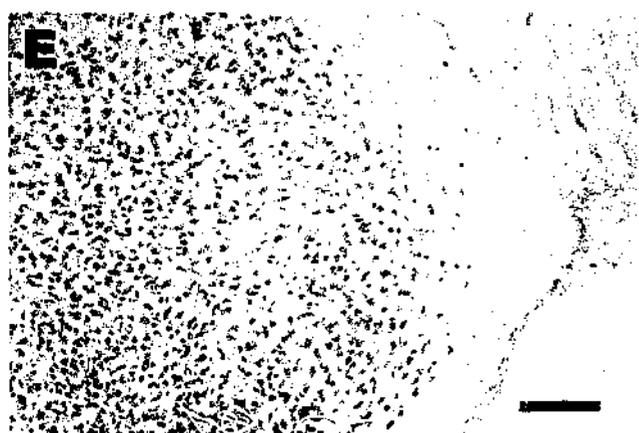
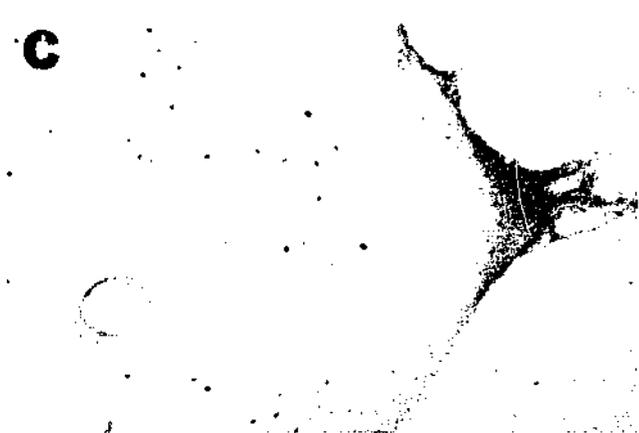
**FIGURE 5.6**

Representative photomicrographs of Fos protein expression in neurons of the supraoptic nucleus (SON) of WKY rats that were microinjected (i.c.v.) with 5 $\mu$ l of saline (n=3; A) or naloxone (100nmol; n=3; B) or WKY subjected to a 60 min restraint session that was preceded by an i.c.v. microinjection of saline (n=4; C) or 100nmol naloxone (n=4; D). Panels A and B demonstrate that a similar level of Fos expression was observed in the SON of unrestrained control rats receiving i.c.v. saline (A) or naloxone (B). A higher number of Fos-positive cells were evident in the SON of rats exposed to restraint (panels C and D) when compared to the unstressed controls depicted in panels A and B. Panel D demonstrates the reduced expression of Fos protein that was observed in the SON of rats that had been pretreated with 100nmol naloxone (i.c.v.) immediately preceding the restraint session when compared to restrained rats receiving i.c.v. saline (C). Furthermore, panels C and D also clearly show that the expression of Fos protein induced by restraint was primarily confined to the magnocellular neurons of the SON. Panel E represents an adjacent section of the SON that was thionin-stained for use as an anatomical reference, while the scale bar represents 75 $\mu$ m.



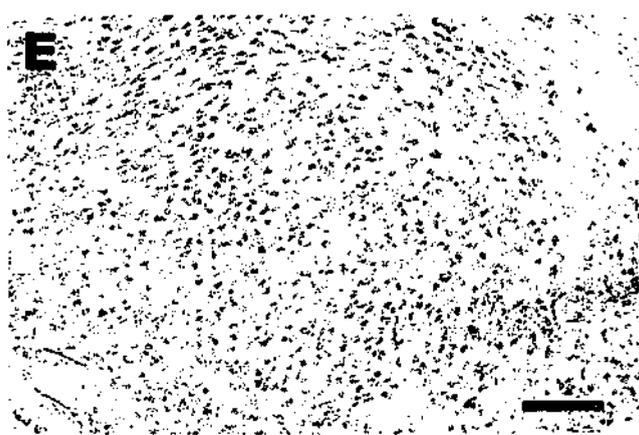
**FIGURE 5.7**

Representative photomicrographs of Fos protein expression in neurons of the medial nucleus of the amygdala (Me) of WKY rats that were microinjected (i.c.v.) with 5 $\mu$ l of saline (n=3; A) or naloxone (100nmol; n=3; B) or WKY subjected to a 60 min restraint session that was preceded by an i.c.v. microinjection of saline (n=4; C) or 100nmol naloxone (n=4; D). Panels A and B demonstrate that a similar level of Fos expression was observed in the Me of unrestrained control rats receiving i.c.v. saline (A) or naloxone (B). A higher number of Fos-positive cells were evident in the Me of rats exposed to restraint (panels C and D) when compared to the unstressed controls depicted in panels A and B. A similar level of Fos protein expression was observed in the Me of rats that had been pretreated with 100nmol naloxone (i.c.v.) immediately preceding the restraint session when compared to restrained rats receiving i.c.v. saline (C). Panel E represents an adjacent section of the Me that was thionin-stained for use as an anatomical reference, while the scale bar represents 150 $\mu$ m.



**FIGURE 5.8**

Representative photomicrographs of Fos protein expression in neurons of the central nucleus of the amygdala (Ce) of WKY rats that were microinjected (i.c.v.) with 5 $\mu$ l of saline (n=3; A) or naloxone (100nmol; n=3; B) or WKY subjected to a 60 min restraint session that was preceded by an i.c.v. microinjection of saline (n=4; C) or 100nmol naloxone (n=4; D). Panels A and B demonstrate that a similar level of Fos expression was observed in the Ce of unrestrained control rats receiving i.c.v. saline (A) or naloxone (100nmol; B). Restraint did not alter the expression of Fos protein in the Ce of WKY rats when compared to unrestrained controls in both treatment groups. In addition, a similar number of Fos-positive cells were visualised in the Ce of restrained rats receiving either i.c.v. saline (C) or naloxone (100nmol; D). Panel E represents an adjacent section of the Ce that was thionin-stained for use as an anatomical reference, while the scale bar represents 150 $\mu$ m.



apparent in specific cell groups. In the PVN, minimal numbers of Fos-positive cells were observed in the magnocellular region, with the majority of Fos-ir detected in cells localised to the pPVN as shown in Figure 5.5C and D. In contrast, a large proportion of magnocellular neurons in the SON contained Fos-ir following exposure to restraint (Figure 5.7C and D).

However, no effect of restraint on Fos production was observed in the Ce (Figure 5.8), ventrolateral PAG (Figure 5.9E–H) and dorsolateral PAG, although it appears that there is a trend towards a decrease in Fos-ir in the Ce. In the ventrolateral PAG, restraint induced a non-significant increase in the number of Fos-positive cells relative to unrestrained, saline-injected rats. The reason for the lack of significance can be attributed to the large variability in data obtained from the cell counts in the ventrolateral PAG in the unrestrained and restrained groups.

The NTS was divided into thirds and classified as caudal, subpostremal and rostral, and comparison of these three subregions revealed that a significantly higher number of Fos-positive cells were present in the subpostremal region of the NTS compared to both rostral and caudal subregions following acute restraint (Figure 5.14 – NTS) ( $P < 0.05$ ; one way ANOVA with post-hoc Student Newman-Keuls test). This is also evident when comparing the expression of Fos-ir in Figure 5.10A and 5.11C, which are representative photomicrographs of Fos-positive cells in the subpostremal and caudal NTS, respectively, of saline-treated rats that were exposed to acute restraint.

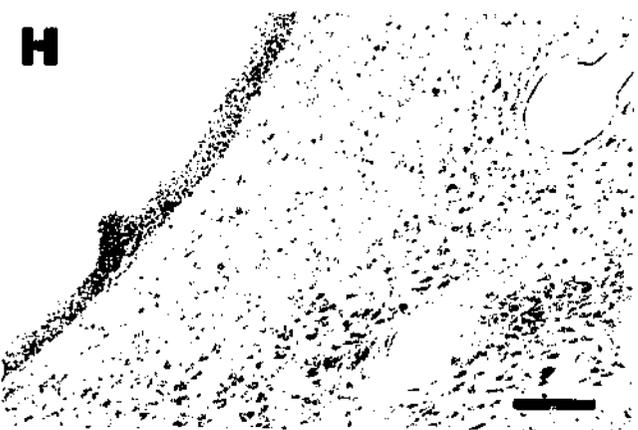
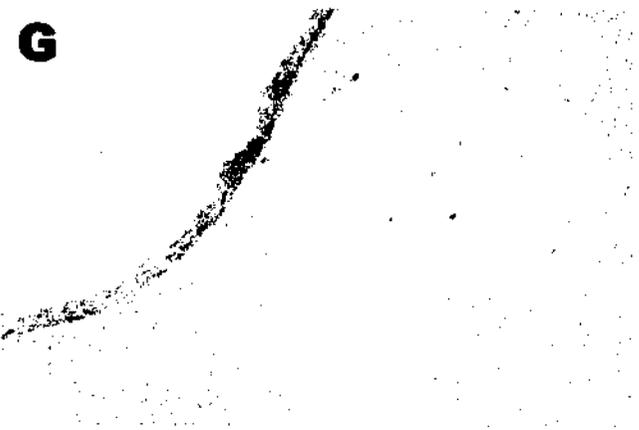
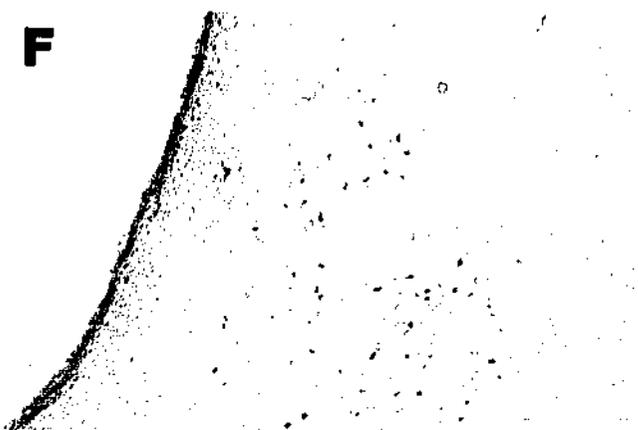
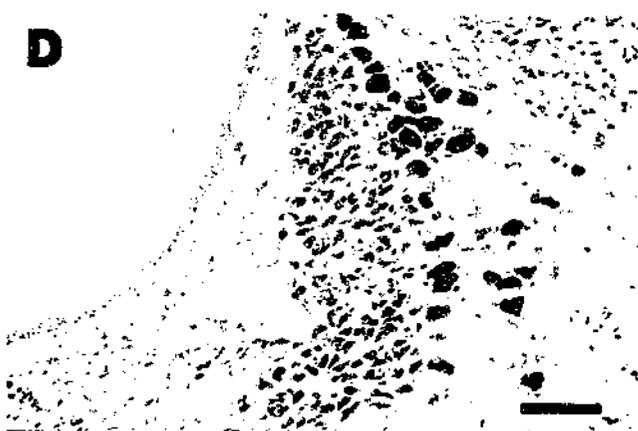
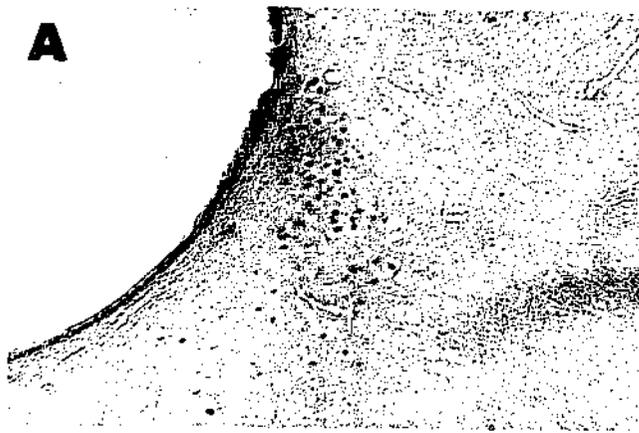
### 5.3.3.3 Effect of *i.c.v.* naloxone on levels of Fos-ir in restrained rats

In addition to the non-stressed rat group (section 5.3.2), naloxone (100nmol; *i.c.v.*) was administered to a separate group of rats immediately preceding exposure to an acute restraint stress paradigm. In these rats, there were no apparent behavioural differences compared to control rats receiving *i.c.v.* saline prior to restraint, with restraint inducing vocalisations, defaecation, urination and increased awareness similar to the control group. To determine the effect of naloxone on the activation of central neurons induced by restraint, Fos-positive cells were quantified in selected nuclei and compared to the results of the control group that was restrained and received *i.c.v.* saline.

Generally, the same pattern of central activation following restraint was observed in naloxone- and saline-treated rats, with significantly increased Fos production observed in regions

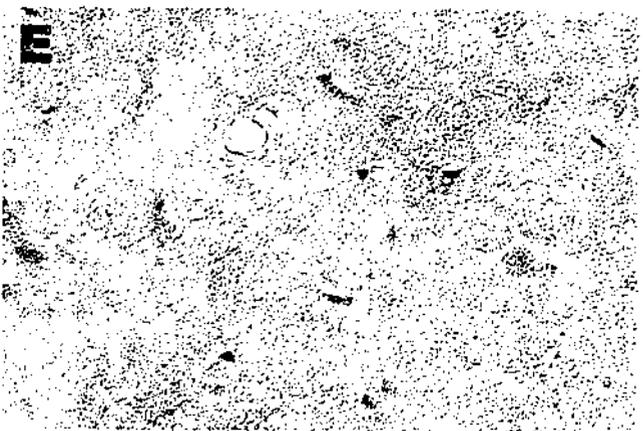
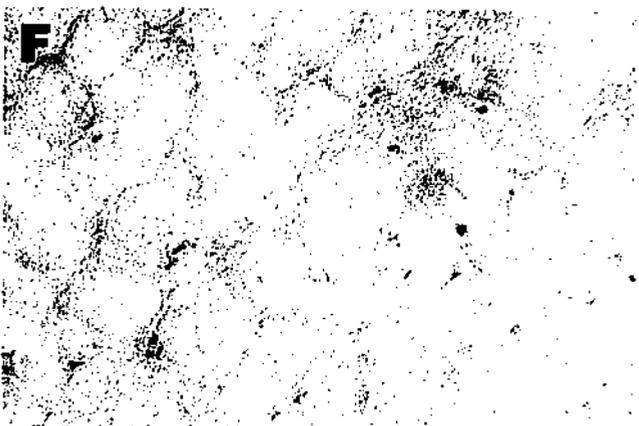
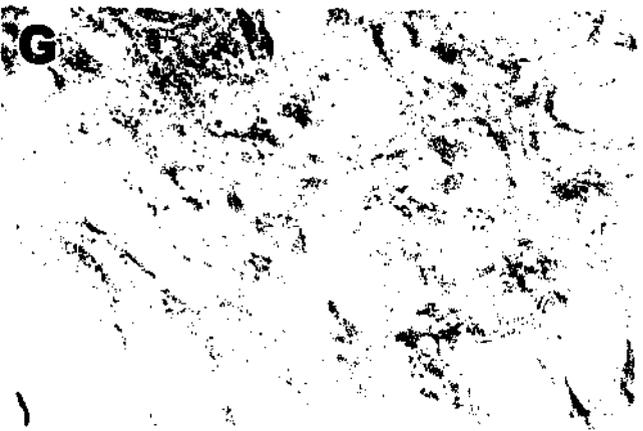
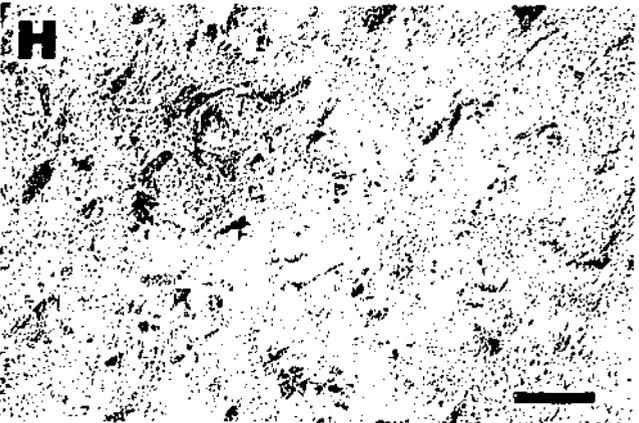
**FIGURE 5.9**

Representative photomicrographs of Fos protein expression in neurons of the locus coeruleus (LC; A, B) and ventrolateral periaqueductal gray (PAG; E, F) of WKY rats subjected to a 60 min restraint session that was preceded by an i.c.v. microinjection of saline (n=4; A, E) or 100nmol naloxone (n=4; B, F). Panels A and B demonstrate that a similar level of Fos expression was observed in the LC of restrained rats receiving i.c.v. saline (A) or naloxone (100nmol; B), while a similar number of Fos-positive cells were also detected in the ventrolateral PAG of restrained rats that were pretreated with i.c.v. saline (E) or naloxone (100nmol; F). Panels C and G shows the low level of Fos expression that was observed in the LC (C) or ventrolateral PAG (G) of unrestrained rats receiving i.c.v. saline (n=3). While not shown in this figure, minimal levels of Fos expression were also observed throughout the LC and ventrolateral PAG of unrestrained rats receiving i.c.v. microinjection of 100nmol naloxone (n=3). Panels D and H represent adjacent sections of the LC (D) and ventrolateral PAG (H) that were thionin-stained for use as an anatomical reference, while the scale bar represents 150 $\mu$ m.



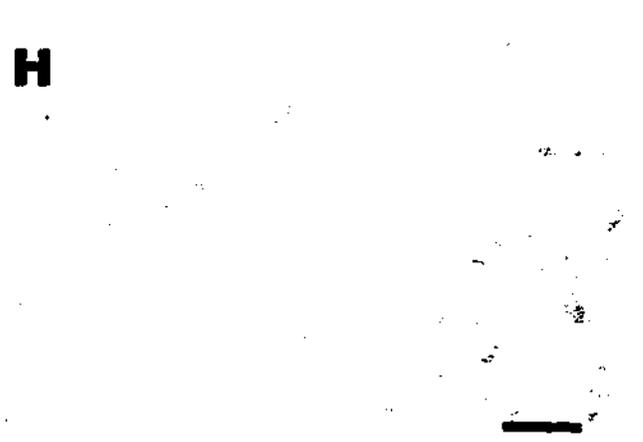
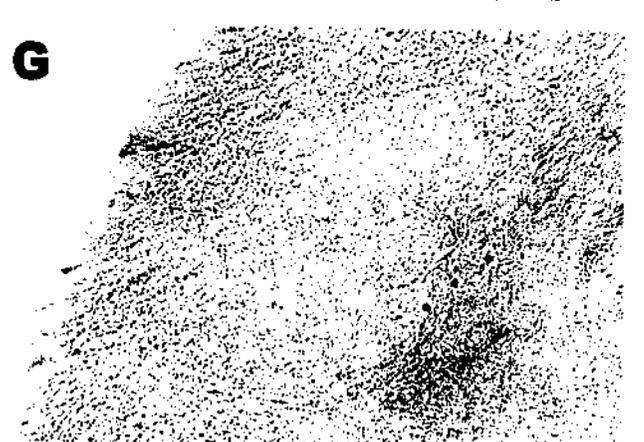
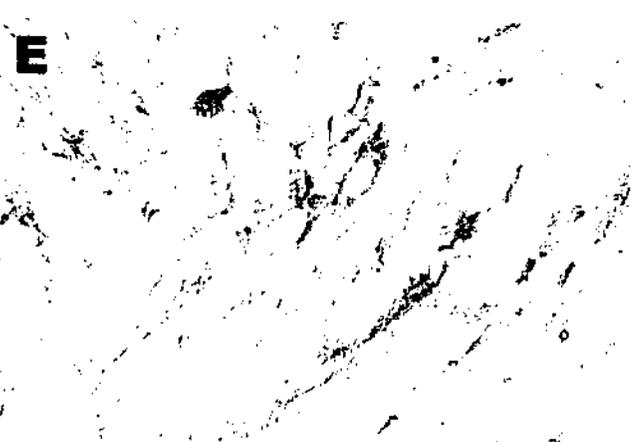
**FIGURE 5.10**

Representative photomicrographs of Fos protein expression in neurons of the nucleus of the solitary tract (NTS) at the level of the area postrema (A, B) and rostral ventrolateral medulla (RVLM; E, F) of WKY rats subjected to a 60 min restraint session that was preceded by an i.c.v. microinjection of saline (n=4; A, E) or 100nmol naloxone (n=4; B, F). Panels A and B demonstrate that a similar level of Fos expression was observed in the subpostremal NTS of restrained rats receiving i.c.v. saline (A) or naloxone (100nmol; B), while a similar number of Fos-positive cells were also detected in the RVLM of restrained rats that were pretreated with i.c.v. saline (E) or naloxone (100nmol; F). Panels C and G shows the low level of Fos expression that was observed in the subpostremal NTS (C) or RVLM (G) of unrestrained rats receiving i.c.v. saline (n=3). While not shown in this figure, minimal levels of Fos expression were also observed throughout the subpostremal NTS and RVLM of unrestrained rats receiving i.c.v. microinjection of 100nmol naloxone (n=3). Panels D and H represent adjacent sections of the subpostremal NTS (D) RVLM (H) that were thionin-stained for use as an anatomical reference, while the scale bar represents 150 $\mu$ m (A-D) or 75 $\mu$ m (E-H).

**A****B****C****D****E****F****G****H**

**FIGURE 5.11**

Representative photomicrographs of Fos protein expression in neurons of the piriform cortex (Pir; A), caudal nucleus of the solitary tract (NTS; C), caudal ventrolateral medulla (CVLM; E) and spinal trigeminal tract (sp5; G) of WKY rats subjected to a 60 min restraint session that was preceded by an i.c.v. microinjection of saline (n=4; A, C, E, G). Fos-positive cells were not quantified in the Pir, CVLM and sp5. Panels B (Pir), D (caudal NTS), F (CVLM) and H (sp5) represent adjacent sections that were thionin-stained for use as an anatomical reference, while the scale bar represents 150 $\mu$ m (A-F) or 75 $\mu$ m (G, H).



such as the PVN, Me, SON, LC, RVLM and NTS compared to the non-restrained controls ( $P < 0.05$ ; student's unpaired  $t$ -test) (Figures 5.8 – 5.11). A comparison of the number of Fos-positive cells in selected brain regions between naloxone- and saline-treated rats revealed that naloxone had no effect on restraint-induced Fos production in the majority of nuclei, including regions such as the Me and LC (Figures 5.12 – 5.13). In the NTS, i.c.v. naloxone had no effect on the expression of Fos protein in restrained rats (Figure 5.14). As with the saline-treated group, the NTS of naloxone-treated restrained rats was divided into 3 sections and compared. The profile and number of NTS cells expressing Fos-ir following restraint was similar between rats receiving i.c.v. saline and naloxone. Thus, the subpostremal NTS contained the highest levels of Fos expression, which was significantly greater than the caudal and rostral ends of the NTS, and this pattern was apparent in both the saline- and naloxone-pretreated rats exposed to acute restraint ( $P < 0.05$ ; one way ANOVA with post-hoc Student Newman-Keuls test) (Figure 5.14).

In the hypothalamic PVN and SON, i.c.v. administration of naloxone significantly altered Fos production induced by restraint. In the PVN, i.c.v. naloxone administration prior to restraint significantly increased (+38%) the number of Fos-positive cells when compared to the saline-treated controls ( $P < 0.05$ ; unpaired student's  $t$ -test) (Figure 5.12). The increased number of cells containing Fos-ir can also be observed in Figures 5.5C and 5.5D, where representative photographs of sections of the PVN obtained from saline (Figure 5.5C) and naloxone (Figure 5.5D) pretreated rats that were exposed to restraint have been presented. Figures 5.5C and 5.5D also show clearly the minimal expression of Fos protein that was observed in the magnocellular subregions of the PVN in both saline- and naloxone-treated rats exposed to restraint.

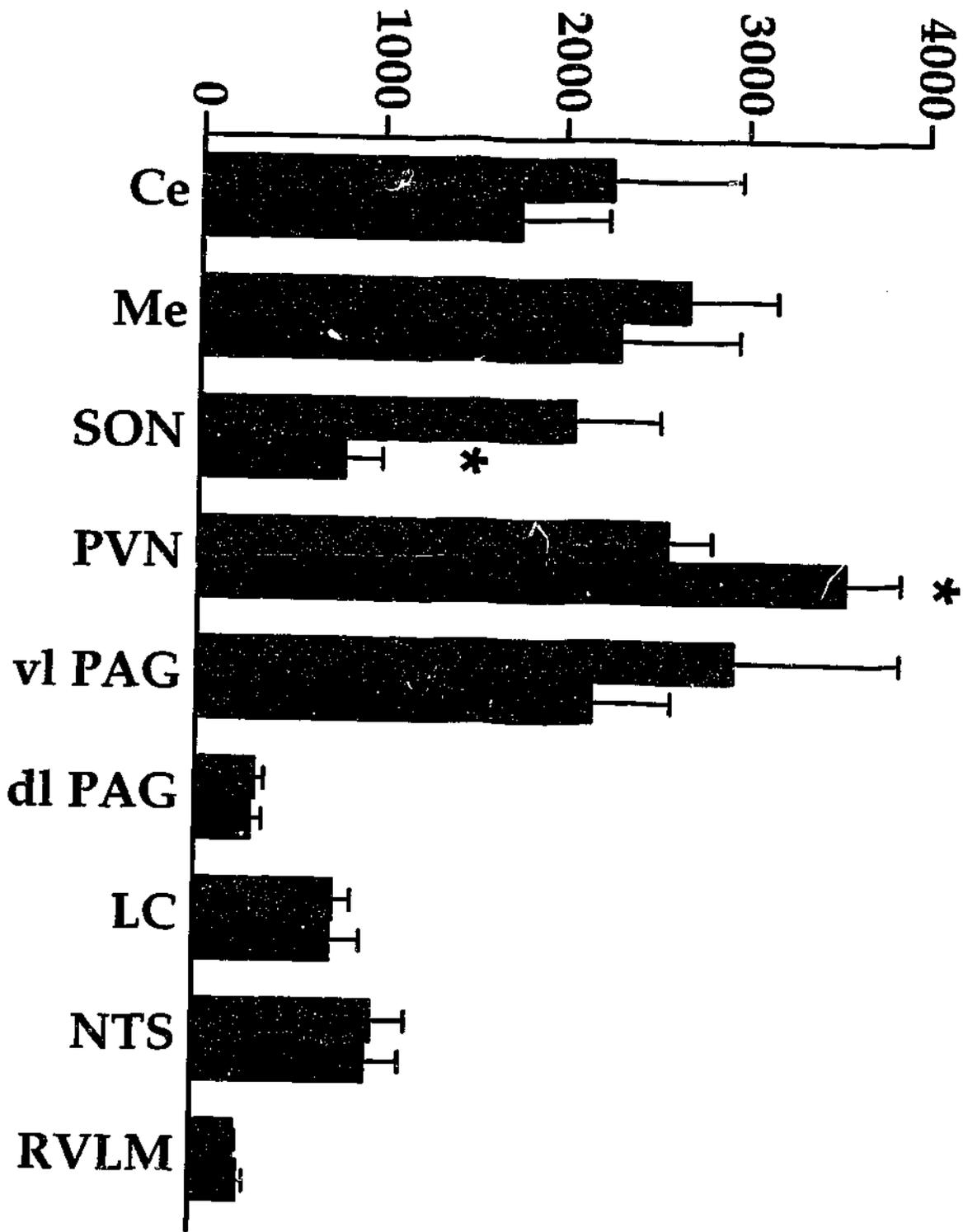
In contrast, the expression of restraint-induced Fos-ir was significantly attenuated (-61%) by i.c.v. naloxone in the magnocellular neurons of the SON ( $P < 0.05$ ; student's unpaired  $t$ -test; two way ANOVA) (Figure 5.12 and 5.13B). The difference in the number of Fos-positive cells in the SON between restrained rats receiving either i.c.v. saline or naloxone can be observed in Figure 5.6C and 5.6D. Furthermore, as shown in Figure 5.13B, significant differences in Fos production could be attributed to specific anatomical locations within the SON ( $P < 0.05$ ; student's unpaired  $t$ -test; two way ANOVA with post-hoc Bonferroni test).

**FIGURE 5.12**

Effect of i.c.v. administration of 100nmol naloxone (n=4; blue columns) or saline (n=4; red columns) on the expression of Fos-positive cells visualised in selected regions of the CNS of WKY rats that had been exposed to a single 60 min period of restraint. Data represent the total number of Fos-positive cells that were quantified for each region, and data are represented as the mean  $\pm$  S.E.M. For abbreviations, refer to page xvii.

\*:  $P < 0.05$ ; unpaired student's *t*-test.

# # Fos-Positive Cells



**FIGURE 5.13**

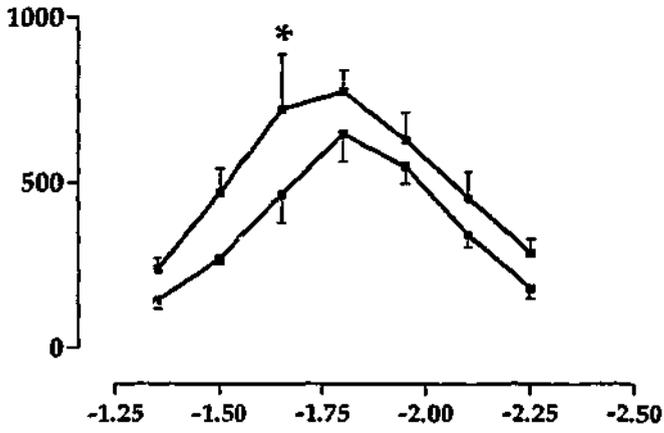
Effect of i.c.v. administration of 100nmol naloxone (n=4; blue) or saline (n=4; red) on the expression of Fos-positive cells visualised in the PVN (A), SON (B), Me (C), LC (D), PAG (E; consisting of the ventrolateral PAG (vlPAG) and dorsolateral PAG (dlPAG)) and VLM (F) of WKY rats that had been exposed to a single 60 min period of restraint. Panels A-F depict the distribution of Fos-positive cells along a rostro-caudal axis within each region. Moreover, each data point represents the average number of Fos-positive cells  $\pm$  S.E.M. contained in 2 consecutive sections obtained from either the naloxone- or saline-pretreated rats that were exposed to restraint (n=4 per group). Note that when collecting sections containing the discrete nuclei included in panels A-F, every third section was removed to use as part of the reference map, and these missing sections were considered when assigning anatomical coordinates for each region. Bregma is measured in millimetres and for abbreviations, refer to page xvii.

†:  $P < 0.05$ ; two way ANOVA comparing the quantity of Fos-positive cells throughout the entire region between saline- and naloxone-treated groups.

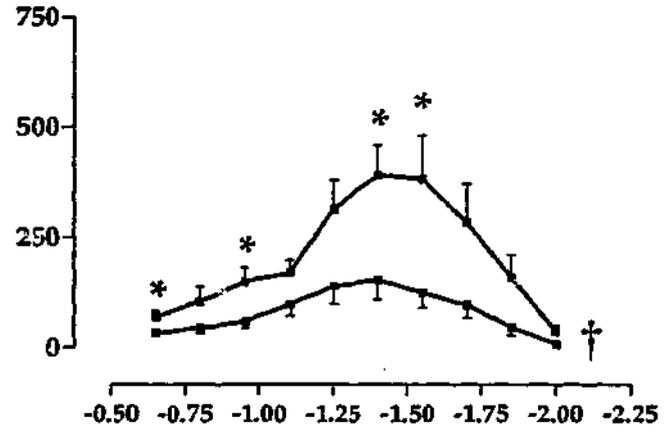
\*:  $P < 0.05$ ; two way ANOVA with post-hoc Bonferroni test comparing the quantity of Fos-positive cells at each anatomical location between saline- and naloxone-treated groups.

# Fos-Positive Cells

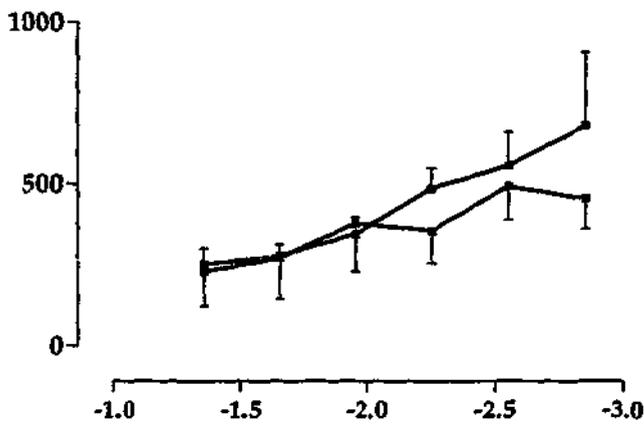
A - PVN



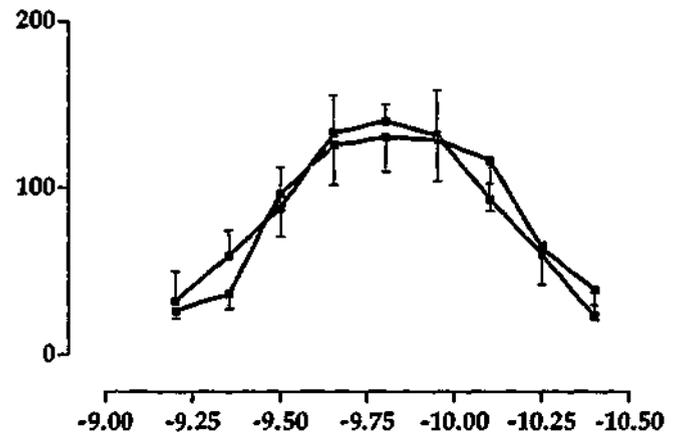
B - SON



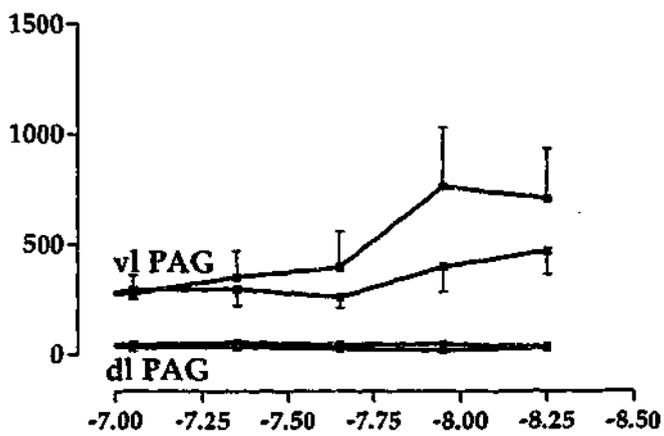
C - Me



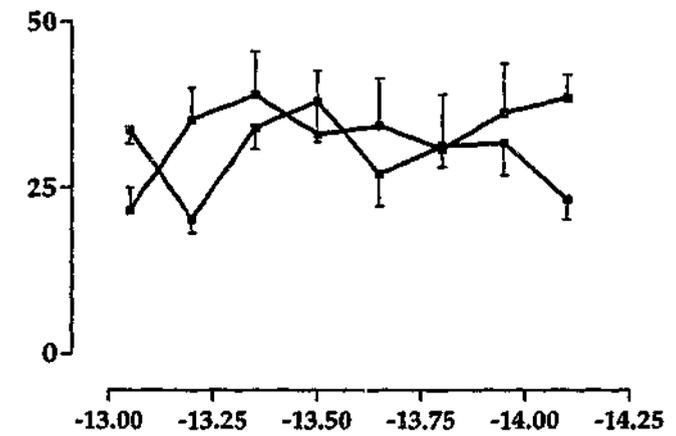
D - LC



E - PAG



F - VLM



Bregma

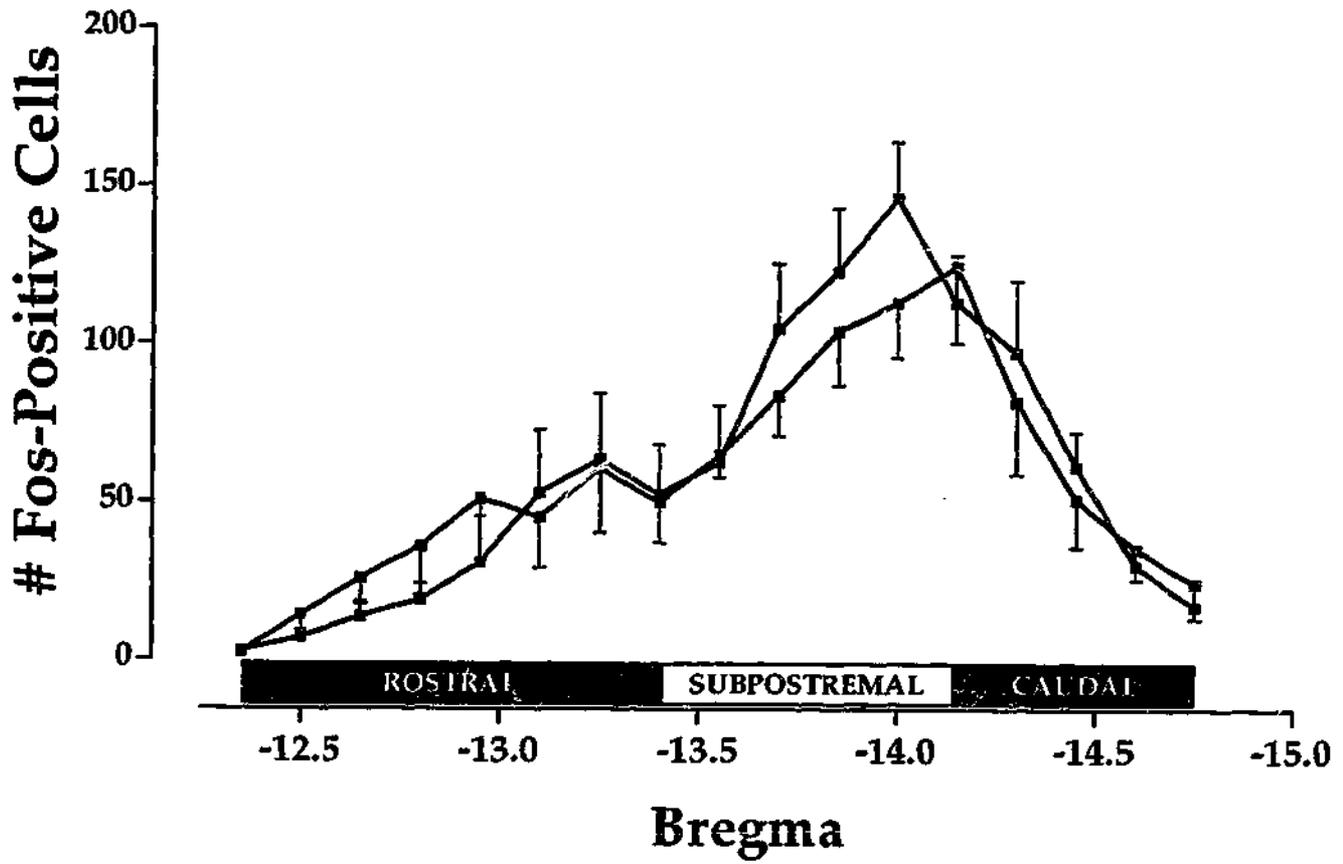
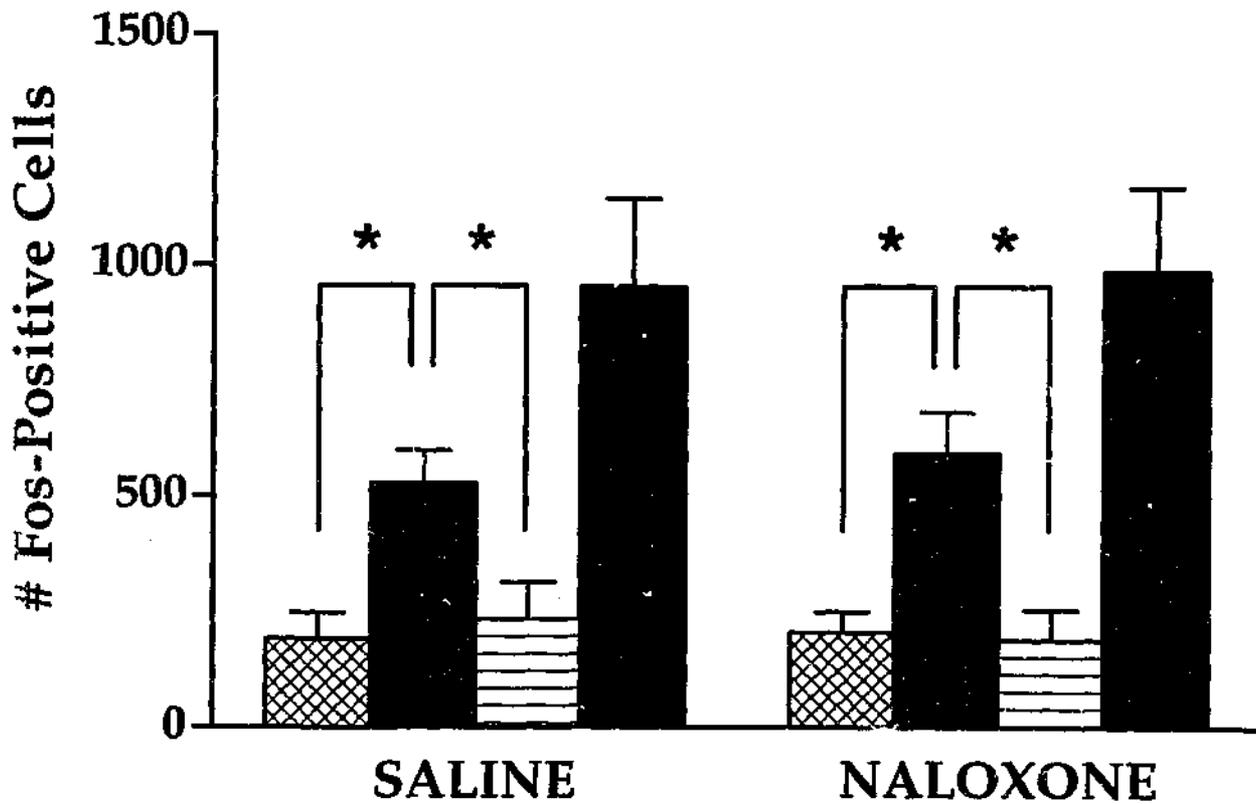
**FIGURE 5.14**

Effect of i.c.v. administration of 100nmol naloxone (n=4) or saline (n=4) on the expression of Fos-positive cells visualised in the nucleus of the solitary tract (NTS) of WKY rats that had been exposed to a single 60 min period of restraint.

**A** - Panel A demonstrates the rostro-caudal distribution of Fos-positive cells throughout the NTS of restrained rats receiving i.c.v. naloxone (blue) or saline (red). Note that each data point represents the average number of Fos-positive cells  $\pm$  S.E.M. contained in 3 consecutive sections of the NTS obtained from either the naloxone- or saline-pretreated rats that were exposed to restraint (n=4 per group). Furthermore, when collecting sections of the NTS to be processed for Fos immunohistochemistry, every fourth section was removed to use as part of the reference map, and these missing sections were considered when assigning anatomical coordinates for the NTS. Bregma was measured in millimetres. No significant difference in the number of Fos-positive cells between rats receiving i.c.v. naloxone or saline was observed at any level of the NTS (two way ANOVA with post-hoc Bonferroni test).

**B** - A comparison of the total number of Fos-positive cells in the different regions of the NTS of rats exposed to restraint and receiving i.c.v. saline or naloxone. The NTS was subdivided into the NTS rostral of area postrema (AP) (hatched blue columns), subpostremal NTS at level of AP (solid blue columns) and NTS caudal of AP (horizontal lined blue columns). The total number of Fos-positive cells in the entire NTS is also included (black columns). In both treatment groups, the highest number of Fos-positive cells were detected in the subpostremal NTS. Data are represented as the mean  $\pm$  S.E.M.

\*:  $P < 0.05$ ; one way ANOVA with post-hoc Student Newman-Keuls test when compared to both the rostral and caudal NTS within the treatment group.

**A****B**

## 5.4 DISCUSSION

The present study investigated the effect of i.c.v. administration of naloxone on central neuronal activation induced by acute exposure to restraint stress. Quantification of the number of Fos-positive cells in selected brain nuclei demonstrated that numerous CNS regions were activated by acute restraint, including the PVN, LC and NTS. Comparison of Fos production in selected regions of naloxone-treated rats with control saline-treated rats revealed that i.c.v. naloxone resulted in a significant increase in the number of neurons activated by restraint in the pPVN. In addition, i.c.v. naloxone significantly suppressed neuronal activation induced by restraint in the SON compared to saline-treated rats. These results demonstrate that endogenous opioids are apparently involved in the modulation of the stress response of central neurons within the PVN and SON. Furthermore, the integral role of both the PVN and SON in the regulation of neuroendocrine function and associated autonomic systems suggests that central opioid-containing neurons may influence the stress response in these systems.

### 5.4.1 I.C.V. SALINE/NALOXONE AND FOS PRODUCTION

The expression of *c-fos* mRNA or Fos protein production in central neurons of the rat have been commonly used indicators of neuronal activation following many different stimuli, including stressors such as restraint, isotonic saline and BP challenges such as haemorrhage and drug-induced hypertension and hypotension (Sharp *et al.*, 1991; Melia *et al.*, 1994; Chen & Herbert, 1995; Dun *et al.*, 1995; Minson *et al.*, 1997; Del Bel *et al.*, 1998; Dayas *et al.*, 1999; McLean *et al.*, 1999). Central neurons are also very sensitive to mild stressors such as handling, i.p. injections and novel environments as shown by increased *c-fos* mRNA and Fos-ir in a variety of central regions (Sharp *et al.*, 1991; Krukoff & Khalili, 1997). In the present study, rats were allowed to habituate and acclimatise to the experimental room for a period of at least 16 hours. On the day of the experiment, animals were not handled during the i.c.v. injection procedure and environmental stimulation such as noise was also kept at a minimal level to reduce the effects of these unwanted stimuli on neuronal activation. As shown in the control group of rats receiving i.c.v. saline alone, the production of Fos protein was minimal in the vast majority of central regions.

In the Ce, there was a relatively high level of Fos-ir in non-stressed i.c.v. saline-treated rats. This can be attributed to a high level of "constitutive" Fos, rather than the stress of the procedure. Dayas and colleagues have demonstrated that during restraint, the Me contains a higher level of Fos production than the Ce (Dayas *et al.*, 1999). Therefore, if these rats were stressed, then the Me should contain more Fos-positive cells than the Ce. However, the Me contained an order of magnitude lower number of Fos-positive cells than the Ce, indicating that it is not the stress of the injection procedure that is activating the Ce neurons. Furthermore, neurons in other central regions of saline-injected rats that have previously been associated with the central stress response, such as the PVN and LC (Chen & Herbert, 1995; Stamp & Herbert, 1999), did not express a level of Fos-ir that was comparable to the level of Fos expression observed in rats exposed to restraint and receiving i.c.v. saline.

The effects of central administration of a 100nmol dose of naloxone on central Fos expression were also investigated. The dose of 100nmol of naloxone was found to have no significant effects on BP in unrestrained rats. Previous studies have shown that i.c.v. administration of naloxone in doses as low as 10nmol and as high as 275nmol can have effects on physiological functions such as food intake and ACTH release (Gaillet *et al.*, 1991; Murase *et al.*, 1996). Thus, a dose of naloxone of 100nmol is well within the range of concentrations of naloxone capable of producing pharmacological effects on various systems. Furthermore, this dose of naloxone has the capacity to antagonise the central receptors belonging to the  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid families, as demonstrated by previous studies where doses of naloxone below 100nmol inhibited effects of morphine, the enkephalins and dynorphin A (1-13) (Bhattacharya *et al.*, 1992; Rabkin, 1993).

In the present study, accurate placement of the guide cannula within the lateral ventricle was confirmed in all rats using the angiotensin drinking test as described in section 5.2.1.2. Microinjection of a 5 $\mu$ l volume containing 100nmol naloxone into the lateral ventricle should be able to access all areas of the rat CNS, including the medulla oblongata, particularly with the 120min time period between i.c.v. naloxone administration and perfusion. A previous study by Reyes and colleagues looked at the changes in Fos expression induced by i.c.v. administration of urocortin, a peptide related to CRF. In this study, the injection volume containing various concentrations of urocortin was 2 $\mu$ l, and they observed increased Fos-ir in neurons of the Ce, PVN, PB and NTS 120min after the i.c.v. microinjection of urocortin (Reyes *et al.*, 2001). This study therefore provides evidence that i.c.v. injections of 2 $\mu$ l and greater volumes can access all levels of the rat CNS, from the forebrain to the medulla oblongata.

A dose of 100nmol did not alter MAP, while higher doses of 250nmol and 350nmol produced dose-dependent increases in MAP. Previous studies have found that i.c.v. and peripheral administration of naloxone did not produce any changes in BP or HR in the rat, although cardiovascular changes have been observed in anaesthetised rats following microinjection of naloxone into particular brain regions, such as the PVN, dorsal hippocampus and RVLM (Sun *et al.*, 1996). In addition, intra-NTS administration of 1nmol naloxone in anaesthetised rats elicits a significant decrease in HR with no change in BP (Gordon, 1990). In contrast, intra-PVN administration of 0.1µmol naloxone in conscious rats produced no change in HR or BP (Kiritsy-Roy *et al.*, 1986). It is therefore unclear what region is mediating the naloxone-induced hypertension seen in the present study, as the studies of Sun and Gordon were conducted in anaesthetised rats. Moreover, there is further evidence that anaesthesia may alter the cardiovascular response to particular agonists, with intra-NTS injection of glutamate producing opposite changes in conscious and anaesthetised rats (Talman *et al.*, 1980; Machado & Bonagamba, 1992). Apart from the Kiritsy-Roy study (Kiritsy-Roy *et al.*, 1986), the cardiovascular effects of naloxone microinjection into discrete central nuclei have been limited, and further studies are therefore required to ascertain whether anaesthesia alters the cardiovascular response following blockade of opioid receptors in the dorsal hippocampus, NTS and RVLM.

In the present study, i.c.v. naloxone did not significantly increase Fos expression in unstressed rats in any central region where Fos-positive cells were quantified, including nuclei such as the PVN, SON, Me and PAG. These results are in agreement with studies investigating the expression of Fos protein in rats receiving central or peripheral administration of naloxone or naltrexone, with no significant changes in the PVN, SON, Me and PAG (Pomonis *et al.*, 1997; Carr *et al.*, 1999; Gestreau *et al.*, 2000). However, there is a trend towards an increase in Fos expression in the Ce and NTS, although this was not significant. In contrast, previous studies of non-selective opioid receptor blockade using naltrexone or naloxone have demonstrated that there is an increased level of Fos-ir in the Ce and NTS, as well as the NAcc, BNST, supramammillary nucleus, RVLM and AP (Kraus *et al.*, 1996; Pomonis *et al.*, 1997; Carr *et al.*, 1999; Gestreau *et al.*, 2000). As outlined in section 5.1, Gestreau and colleagues investigated the changes in central Fos production produced by i.v. administration of 5.5µmol/kg naloxone, and reported elevated levels of Fos-positive cells in the Ce, supramammillary nucleus, AP, NTS and RVLM of rats receiving i.v. naloxone compared to rats receiving i.v. saline (Gestreau *et al.*, 2000). The observation that i.v. administration of naloxone-methiodide, a naloxone analogue

that does not cross the blood-brain barrier, did not produce any significant changes in Fos-ir in any central region suggests that the increase in Fos-positive cells observed following i.v. naloxone are due to a direct action on central opioid receptors. However, it must be noted that a comparison of the number of Fos-labelled cells in the supramammillary nucleus of naloxone and naloxone-methiodide treated rats found that there was no significant difference between groups (Gestreau *et al.*, 2000).

Differences in experimental procedure may account for the variation in results between the study by Gestreau and co-workers and the present investigation. The major difference is the route of administration of naloxone, with i.c.v. used in the present study compared to a peripheral route (i.v.) used by Gestreau and colleagues (Gestreau *et al.*, 2000). The Gestreau study, however, did publish data using naloxone methiodide to attribute the effects of peripheral administration of naloxone to central regions. However, peripheral (i.v. and i.p.) administration of naloxone in unstressed rats has been shown to cause some physiological effects. At the same dose of 5.5  $\mu\text{mol/kg}$  (i.v. or i.p.) or lower, naloxone suppresses the release of prolactin, increases plasma oxytocin and vasopressin levels and induces variable changes in plasma corticosterone while no changes in BP, HR, plasma ACTH or hypothalamic CRF activity were detected (Hashimoto *et al.*, 1986; Iyengar *et al.*, 1986; Buckingham & Cooper, 1987; Florentino *et al.*, 1987; Shibuki *et al.*, 1988; Cover & Buckingham, 1989; Xu & McCann, 1989; Yamada *et al.*, 1989b). Thus, it is possible that i.v. naloxone may have altered a neuroendocrine system such as those described above at a peripheral site of action, which may have ultimately produced the Fos-ir increases that were detected in the CNS.

Despite their results, it is not clear what concentration of naloxone reaches and remains within the CNS following i.v. administration of a 5.5  $\mu\text{mol/kg}$  dose of naloxone. The resultant CNS concentration following peripheral administration of naloxone appears to be important, as i.p. injection of a lower dose of naloxone (2.75  $\mu\text{mol/kg}$ ) increased Fos-positive cells in the Ce only, and not in the VLM, NTS, PVN or SON (Pomonis *et al.*, 1997). Thus, the higher dose of naloxone used by Gestreau and colleagues may be antagonising a larger population of opioid receptors that consists of all subtypes, while the present study and that of Pomonis and colleagues may not be producing the same magnitude of antagonism at each opioid receptor subtype.

Another difference between the present study and that of Gestreau was the pre-injection handling procedure. In the present study, rats were not handled prior to the i.c.v. injection, as the

injector could be placed in the guide without disturbing the rat. The low level of stress induced by the injection procedure, environment and surroundings is demonstrated by the low level of Fos protein in regions activated by stress, such as the Me, PVN and LC. Moreover, when the expression of Fos-ir is compared between animals receiving an i.c.v. injection and rats exposed to restraint, it is clear that restraint is a stressor of a much larger magnitude (see following section).

Central administration (i.c.v.) of naltrexone (265nmol) was found to significantly increase Fos production in the Ce, BNST and NAcc when compared to rats receiving i.c.v. saline (Carr *et al.*, 1999), while a similar study where naltrexone (16 $\mu$ mol/kg) was administered peripherally (s.c.) also reported increased Fos-ir in the Ce and BNST compared to saline-treated rats (Carr *et al.*, 1998). The difference in results could be attributed to one of a number of protocol differences between the studies by Carr and colleagues (Carr *et al.*, 1998; Carr *et al.*, 1999) and the present study, keeping in mind that the level of Fos production in the BNST and NAcc was not investigated in the present thesis. Firstly, naltrexone has a slightly longer serum half-life than naloxone when compared in the dog (85min [naltrexone] vs 71min [naloxone]) (Pace *et al.*, 1979), and when combined with the higher concentration of naltrexone used in the Carr laboratory studies, naltrexone may be blocking a higher proportion of opioid receptors for a longer period of time. Secondly, the delay between naltrexone administration and perfusion in the Carr studies was 90 min, while the present study used an injection-perfusion time period of 120 min. Thus, it is possible that the neuronal effects resulting from non-selective opioid receptor blockade may be larger at 90 min rather than 120 min after the injection of the antagonist.

A different approach was used to investigate the basal activity of the opioid system by Kraus and colleagues (Kraus *et al.*, 1996). They investigated changes in 2-deoxy-D-[1- $^{14}$ C]glucose ([ $^{14}$ C]-2-DG) concentrations in discrete brain regions following a 2.75 $\mu$ mol/kg i.v. injection of naloxone or saline, with the levels of [ $^{14}$ C]-2-DG used as an indicator of local cerebral metabolic rate for glucose. While this technique allows detection of drug-induced increases in activity in a particular region, it also provides an indication of any decreases in neuronal activity, information that Fos immunohistochemistry cannot always provide. Comparison of the effects of naloxone and saline in 84 brain regions revealed that i.v. naloxone increased the activity of neurons within the Ce, while it produced a decrease in LC neurons (Kraus *et al.*, 1996). However, they did not collect sections in the medulla oblongata, so it is not known whether significant increases in metabolic activity existed in the AP, NTS and RVLM, regions where increased Fos-ir has been

found in rats treated with central or peripheral naloxone (Gestreau *et al.*, 2000). However, Kraus and colleagues did not find any significant effects in the BNST and NAcc as reported in the later studies by Carr and Gestreau and co-workers (Carr *et al.*, 1998; Gestreau *et al.*, 2000).

In addition to the increase in [ $^{14}\text{C}$ ]-2-DG concentration in the Ce of rats receiving s.c. naloxone (Kraus *et al.*, 1996), the studies of Gestreau and Carr (Carr *et al.*, 1998; Carr *et al.*, 1999; Gestreau *et al.*, 2000) described above found an increase in Fos-ir in the Ce following central or peripheral administration of naloxone or naltrexone. The present study observed an increasing, but non-significant, trend in Fos-containing cells in the Ce, a difference that may be explained by a different protocol for quantification of Fos-positive cells. The studies conducted by the Carr and Gestreau laboratories utilised a computer analysis package that automatically counted Fos-positive cells that conformed to pre-determined criteria for size, shape and density. While this method permits a rapid quantification of Fos-positive cells, I found that it often excluded very lightly stained Fos-ir cells within a particular region. Furthermore, a computer-assisted counting procedure requires the "capture" or saving of the image which only represents one focal plane of the brain slice. The brain sections used in the present study are 50 $\mu\text{m}$  thick, so that cells are present at different levels of the section. Cells that are slightly above or below the focal plane appear blurred or dull, and the criteria set for computer analysis may not include these Fos-positive cells that are just outside the focal plane. In the present study, Fos-positive cells were counted manually through all focal planes of the 50 $\mu\text{m}$  section, with cells deemed to contain Fos-ir if they could be clearly discerned from the background. Thus, larger numbers of Fos-positive cells have the potential to be counted in the present study using this method. While many regions were found to contain a low basal level of Fos production using this counting protocol, the Ce exhibited a relatively high basal level of Fos expression. The studies by Carr and colleagues reported basal levels of Fos-ir in the Ce of saline-treated rats of between 10 and 20 Fos-positive cells per 40 $\mu\text{m}$  section (Carr *et al.*, 1998; Carr *et al.*, 1999), while the present study found that an average of 80 Fos-positive cells could be counted in the Ce of unstressed rats over a range of 13 sections of 50 $\mu\text{m}$  thickness. It therefore appears that computer-assisted quantification of Fos-positive cells only includes cells with moderate to strong Fos-ir. As the present study also counted cells with light Fos-ir, then it is possible that any increase in number of Fos-containing cells with strong Fos-ir in the Ce induced by central or peripheral naloxone administration would not have been detected.

## 5.4.2 RESTRAINT AND FOS PRODUCTION

The pattern of central neuronal activation induced by a restraint stimulus has been reported in many studies, with regions such as the septum, hippocampus, various subregions of the cortex, hypothalamus (PVN, SON, DM), amygdala (Me, Ce), PAG, LC, RVLM, CVLM and NTS in the conscious rat activated by this particular psychological stressor (Melia *et al.*, 1994; Chen & Herbert, 1995; Cullinan *et al.*, 1995; Krukoff & Khalili, 1997). In the present study, the number of Fos-ir cells was found to be increased in many of these central regions in rats exposed to an acute restraint stimulus. Specifically, Fos-positive cells were quantified in the PVN, SON, Me, Ce, LC, ventrolateral and dorsolateral PAG, NTS and RVLM, and all of these regions, with the exception of the dorsolateral PAG and Ce, exhibited an increased expression of Fos protein in response to the restraint stimulus when compared to unrestrained rats receiving an i.c.v. saline injection. Thus, the present findings support the results of previous studies and confirmed the effectiveness of the current restraint paradigm. Furthermore, the same restraint paradigm has been demonstrated to induce neurochemical and behavioural changes as detailed in chapters 3 and 4 and previously published studies (McDougall *et al.*, 2000).

In the present study, the activity of neurons within the Ce of rats exposed to acute restraint were not significantly altered, a result that is in contrast to previous findings that have reported various degrees of neuronal activation within the Ce following restraint stress (Honkaniemi *et al.*, 1992a; Chen & Herbert, 1995; Cullinan *et al.*, 1995; Bhatnagar & Dallman, 1998; Dayas *et al.*, 1999). There may be a number of plausible explanations for this discrepancy, remembering that results from previous chapters have shown that restraint produced neurochemical alterations in prepro-GAL mRNA expression and GAL receptor density in the Ce (see Chapters 3 and 4). Firstly, restraint stress may induce a net inhibition of Ce neurons. As a consequence, there would be either no change or a decrease in neuronal Fos protein production in this region. GABA is one of the principal inhibitory neurotransmitters in the CNS, and neurochemical markers for GABA, such as glutamic acid decarboxylase (GAD)- and GABA-positive neurons and GABA<sub>A</sub> receptors have been detected within this amygdaloid nucleus (Nitecka & Ben-Ari, 1987; Sun & Cassell, 1993; Kunkler & Hwang, 1995; Veinante *et al.*, 1997). In addition, *c-fos* mRNA or Fos protein may not be the most appropriate marker for neuronal activation in this region, with other immediate early genes such as *c-jun* and *zif/268* used in previous studies as alternative markers of neuronal activation. However, Cullinan and colleagues (Cullinan *et al.*, 1995) reported an increase in *c-jun* and *zif/268* mRNA in the Ce 30 min after restraint stress,

indicating that restraint stress can induce increases in production of a variety of immediate early genes and their protein products. Lastly, the time course used in the current study (60 min restraint followed by 60 min rest prior to perfusion) may not be optimal for the detection of restraint-induced changes in Fos protein production in the Ce. Rapid increases in Fos-positive cells in the Ce may peak during the first few min of restraint, followed by a slow decline to levels of Fos production that are not distinguishable from non-stress levels. A study that determines Fos protein levels at various time points following initiation of restraint would provide a clearer explanation of the response of the Ce to this stress paradigm.

Comparing the levels of Fos induction in the Me and Ce share some correlations with a previous study conducted by Dayas and colleagues (Dayas *et al.*, 1999). In their study, rats were exposed to a 15 min restraint stimulus and the change in number of Fos-positive cells in the Ce and Me were compared. As in the present study, Dayas and colleagues found that restraint produced a larger increase in the number of Fos-positive cells in the Me compared to the Ce. Moreover, they also found that ablation of the Me, but not the Ce, suppressed the activation of the oxytocin-containing cells in the PVN and SON (Dayas *et al.*, 1999). The present study therefore provides further evidence that the cells of the Me, but not the Ce, are preferentially activated by a restraint stimulus. However, the relationship between neuronal activation in the Me and the physiological response to restraint was not investigated in the present study. A further study could extend the current findings and the work of Dayas and colleagues by lesioning the Me and observing any effects on plasma hormone levels, particularly oxytocin, during the response to restraint and other stressors.

### 5.4.3 OPIOIDS AND THE CENTRAL RESPONSE TO RESTRAINT

To study the role of the opioid system in the neural response to restraint, central opioid receptors were inhibited by a global (i.c.v.) microinjection of the non-selective opioid receptor antagonist naloxone, and the subsequent effects on Fos production in selected regions of the CNS were quantified. As such, the current study represents a novel report of central opioid inhibition and its modulatory effects on stress-induced neuronal activity. Naloxone had no significant effect on restraint-induced Fos levels in pontine or medullary regions, including the PAG, LC, RVLM and NTS. This result is interesting, given that earlier reports have found that central (i.c.v.) or systemic administration of naloxone suppressed the elevation in BP caused by

air-jet stress, social deprivation, inescapable shock and cold stress, possibly through  $\mu$ -opioid receptors (Florentino *et al.*, 1987; Kapusta *et al.*, 1989; Jiménez *et al.*, 1990). While these stressors may elicit slightly different neural responses compared to restraint, all of these psychological stressors have been shown to increase BP as part of the physiological stress response. Therefore, as neuronal activation resulting from restraint was not altered by naloxone in the major cardiovascular regulatory centres located in the brainstem (NTS, RVLM), the opioid-mediated modulation of stress-induced hypertension may not be mediated by these regions, but via an alternative nucleus which may be located in the forebrain, such as the PVN (Shafton *et al.*, 1998; Pyner & Coote, 2000). To further investigate the association between central opioid receptors and restraint-induced hypertension, the first group of experiments should study the modulatory effects of i.c.v. naloxone on BP in rats exposed to restraint stress. From this point, many different directions can be taken, including observation of neuronal activation and BP status following the central administration of more selective opioid antagonists or antisera directed at opioid receptors ( $\mu$ ,  $\delta$ ,  $\kappa$ ) or peptides (Met-enkephalin, Leu-enkephalin,  $\beta$ -endorphin).

In contrast to medullary and pontine structures, the response to restraint exhibited by the hypothalamic PVN and SON was altered by prior administration of naloxone. In the PVN, a significant increase in the number of Fos-positive cells was detected in naloxone-treated rats compared to rats receiving i.c.v. saline, indicating that i.c.v. naloxone increased the level of neuronal activation in this nucleus. The majority of Fos-positive neurons activated by restraint in both the naloxone- and saline-treated rats were located in the pPVN. Neurons within the pPVN project to the anterior pituitary and portal vascular system and have been shown to contain CRF (Swanson *et al.*, 1983). Under resting conditions, pPVN CRF-containing neurons contain minimal amounts of vasopressin, with vasopressin levels increasing markedly in these neurons following exposure to various stimuli including adrenalectomy and osmotic and psychological stressors (Sawchenko *et al.*, 1984; Bartanusz *et al.*, 1993; Kovács, 1998). Furthermore, vasopressin acts synergistically with CRF to increase the release of ACTH from the anterior pituitary (Gillies *et al.*, 1982). Opioids and their precursors such as prepro-ENK mRNA and Met-enkephalin have also been colocalised with CRF and vasopressin in neurons of the pPVN (Hökfelt *et al.*, 1983; Ceccatelli *et al.*, 1989b; Sakanaka *et al.*, 1989; Larsen & Mau, 1994). In addition, dense levels of  $\kappa$ -opioid receptor mRNA expression and  $\kappa$ -opioid receptors have been detected in the pPVN, with low amounts of  $\mu$ - and  $\delta$ -opioid receptor mRNA also found in this region (George *et al.*, 1994; Mansour *et al.*, 1994a; Mansour *et al.*, 1994b). Despite a paucity of

autoradiographic studies that have detected populations of  $\mu$ -opioid receptors in the pPVN (Mansour *et al.*, 1994a; Mansour *et al.*, 1994c), functional reports demonstrate that opioid receptors that have a high affinity for  $\mu$  selective agonists are indeed present within the PVN (Kiritsy-Roy *et al.*, 1986; Bachelard & Pitre, 1995). Thus, there is a large amount of evidence suggesting that opioids may be able to regulate the activity of pPVN neurons and associated neuroendocrine function.

The pPVN has a role as one of the final CNS relay stations responsible for integrating and translating central neuroendocrine outputs, and it has been shown to receive dense afferent inputs that contain opioids originating from regions involved in stress, cardiovascular and visceral regulation and neuroendocrine status. Regions such as the lateral reticular nucleus, medial POA, DM, lateral septum, ARC, paragigantocellularis reticular nucleus and NTS have been shown to send efferent enkephalin-containing projections to the pPVN (Beaulieu *et al.*, 1996). POMC-containing neurons in the ARC also project to the PVN (Baker & Herkenham, 1995). In addition, dynorphin-containing projections to the pPVN may originate from regions such as the SON, Ce, VMH, DG, PB, NTS and spinal cord (Swanson & Sawchenko, 1983; Code & Fallon, 1986; Mansour *et al.*, 1994b). Considering that the pPVN is an important link between the CNS and neuroendocrine system, it is not surprising that various stimuli induce alterations in the activity and neurochemistry of neurons within this region. Different stressors, including restraint, have increased levels of transcripts encoding CRF, vasopressin, the  $\kappa$ -opioid receptor and prepro-ENK in the pPVN (Bartanusz *et al.*, 1993; Larsen & Mau, 1994; Yukhananov & Handa, 1996), demonstrating that opioid neurochemistry within the pPVN can be altered by different stressors.

The results of the present study, where inhibition of opioid receptors during exposure to restraint caused a significant increase in Fos production in the pPVN, suggests that opioids released in the pPVN as a consequence of restraint stress result in a net inhibition of neuronal activity. This opioid pathway does not appear to be tonically active, as an increase in Fos-positive cells was not observed in the pPVN of unrestrained rats that also received i.c.v. naloxone. Thus, blocking naloxone-sensitive opioid receptors suppresses this net inhibition and causes an increase in the number of neurons activated by restraint. The functional and physiological consequences of a release of the opioid "brake" in the pPVN during stress are widespread.

The HPA axis is one physiological system that may exhibit changes in the stress response induced by i.c.v. naloxone. While there have been no studies investigating the effects of intra-pPVN administration of opioids on the activity of the HPA axis during stress, central (i.c.v.) administration of opioids have elicited a variety of changes in the HPA axis. Systemic or i.c.v. administration of naloxone in rats exposed to a 10 or 15 min restraint stress did not modify the stress-induced elevation in plasma corticosterone (Herbert & Howes, 1993; Gadek-Michalska *et al.*, 1997b). However, results such as the augmentation of restraint-induced release of plasma corticosterone induced by i.c.v. Met-enkephalin and the attenuation of restraint-induced plasma ACTH levels by a  $\beta$ -endorphin antiserum suggests that opioids may activate the HPA axis during stress (Yamauchi *et al.*, 1997). Furthermore, opioids selective for each receptor subtype can also increase the activity of the HPA axis in the resting state (Iyengar *et al.*, 1986; Eisenberg, 1994; Gadek-Michalska & Bugajski, 1996). As such, the role of the central opioid system in regulating HPA axis activity may depend on the stress and its exposure time, with additional opioid regulatory influences recruited during periods of chronic restraint/stress. Therefore, changes in the activity of the HPA axis (i.e. plasma levels of corticosterone or ACTH) in naloxone-treated rats exposed to the present 60 min restraint paradigm would need to be investigated in future experiments.

A function of the PVN that is starting to receive more attention involves the control of sympathetic outflow through descending projections originating in the pPVN. The early neuroanatomical tracing studies of the PVN by Swanson and colleagues demonstrated the existence of spinally-projecting neurons (Swanson & Kuypers, 1980). More recently, efferents of the pPVN have been shown to terminate in close proximity to sympathetic preganglionic neurons in the IML that project to the stellate ganglion and adrenal medulla (Ranson *et al.*, 1998; Motawei *et al.*, 1999). The pPVN also projects to the RVLM, and terminals of these projections are in close association with the cell bodies of the spinally-projecting neurons of the RVLM (Shafton *et al.*, 1998; Pyner & Coote, 1999; Pyner & Coote, 2000). In addition, Shafton and colleagues and Pyner and Coote showed that the pPVN sends collateral projections to the RVLM and spinal cord (Shafton *et al.*, 1998; Pyner & Coote, 2000). These neuroanatomical studies provide clear evidence that the pPVN has the potential to regulate sympathetic outflow, either at the level of the RVLM, spinal cord or both.

Functional studies have previously demonstrated that microinjection of various agonists, including the  $\mu$ -opioid agonist DAGO, into the PVN of conscious rats can elicit increases in BP, HR and catecholamine release, as well as changes in blood flow in peripheral vascular beds

(Appel *et al.*, 1986; Bachelard & Pitre, 1995). However, microinjection of naloxone into the pPVN of unstressed rats at a dose of 0.1nmol had no significant effect on plasma catecholamine concentration, HR or BP, suggesting that the opioid-mediated influence on the sympathoadrenal system at the level of the PVN may not be tonically active (Kiritsy-Roy *et al.*, 1986). While the opioids in the PVN may not be important regulators of blood pressure in the resting state, studies in stressed rats have demonstrated that the opioids can regulate sympathetic outflow at the level of the PVN. The plasma levels of adrenaline during stress can be regulated by opioids within the pPVN, with intra-PVN naloxone potentiating the elevation of plasma adrenaline, but not NAdr, in response to restraint (Kiritsy-Roy *et al.*, 1986). In addition, Kiritsy-Roy and colleagues found that microinjection of naloxone into the PVN of restrained rats had no effect on restraint-induced tachycardia or hypertension (Kiritsy-Roy *et al.*, 1989). While intra-PVN microinjection of the selective  $\mu$  agonist DAGO had no effect on plasma adrenaline or NAdr levels in restrained rats, it did facilitate the recovery of HR towards resting levels during exposure to restraint (Kiritsy-Roy *et al.*, 1986). These studies therefore suggest that opioid-containing projections to the PVN can regulate neuronal activity during stress, which may result in changes in sympathoadrenal system indices, such as plasma catecholamine concentrations. Future studies could investigate the connections of the PVN with the RVLM and/or the spinal cord, and how these pathways activate or modulate the sympathoadrenal system during the response to stressors such as restraint.

In the present study, i.c.v. naloxone caused a suppression of neuronal activity in the SON of rats exposed to acute restraint. Thus, in contrast to the pPVN discussed earlier, opioids appear to activate SON neurons during restraint. Within the SON, high levels of proDYN mRNA,  $\kappa$ -opioid receptor mRNA and  $\kappa$  receptors have been detected, with low levels of  $\mu$ - and  $\delta$ -opioid receptor mRNA also present throughout the magnocellular SON (Mansour *et al.*, 1994a; Mansour *et al.*, 1994b). However, Harlan and colleagues (Harlan *et al.*, 1987) did not detect any prepro-ENK mRNA in the SON, while mature  $\mu$  and  $\delta$ -opioid receptors have also been difficult to detect (Mansour *et al.*, 1987). Many dynorphin-positive SON neurons also contain vasopressin and GAL, and in addition, dynorphin is colocalised with oxytocin, TH and CRF in a smaller proportion of SON neurons (Meister *et al.*, 1990b). The source of opioid-containing input to the SON is unclear, but regions such as the anterior OB, septal nuclei, Ce, DM, LC, lateral PB, VLM and NTS have all been shown to project to the SON (Anderson *et al.*, 1990; Petrov *et al.*, 1994).

Within the SON, magnocellular neurons were activated by restraint stress in both the naloxone- and saline-treated rats in the current study. This complements an earlier study that also reported increased *c-fos* mRNA expression in oxytocin-containing magnocellular neurons of the SON after restraint stress (Miyata *et al.*, 1995). Restraint and osmotic stressors increased the expression of oxytocin and proDYN mRNA in the SON (Lightman & Young, 1987; Giovannelli *et al.*, 1990; Young & Lightman, 1992), while SON vasopressin mRNA expression was increased selectively by osmotic stimuli, but not restraint (Lightman & Young, 1987; Herman, 1995). In addition, Fos-ir was increased in vasopressin neurons in the SON after hypotensive haemorrhage in rats (Smith *et al.*, 1995).

The magnocellular neurons of the SON that contain either oxytocin or vasopressin are known to project to the posterior pituitary and these two hormones are released from the axons of their respective neurons into the circulation (Zimmerman *et al.*, 1984). A variety of stimuli can increase the release of oxytocin and vasopressin, with elevated plasma levels of oxytocin observed following exposure to psychological (restraint) and physical (hypertonic saline) stressors, while plasma vasopressin concentrations are primarily increased by stimuli altering the osmotic properties of the blood (Hashimoto *et al.*, 1989b; Aguilera & Kiss, 1993; Jezova *et al.*, 1995; Kadakaro *et al.*, 1995; Kjaer *et al.*, 1995b). The presence of dynorphin and Leu-enkephalin in these neurohypophysial SON neurons, particularly with the colocalisation of vasopressin with dynorphins in the same secretory granules and oxytocin and vasopressin with enkephalins in terminals, suggests that opioids may regulate the release of oxytocin and vasopressin in the posterior pituitary (Martin & Voigt, 1981; Molineaux *et al.*, 1984; Quinn & Weber, 1986).

Later studies have supported this hypothesis, with systemic naloxone increasing the basal release of oxytocin (Shibuki *et al.*, 1988; Leng *et al.*, 1992) and oxytocin release stimulated by i.p. or intra-SON hypertonic saline (Carter & Lightman, 1987; Munro *et al.*, 1994). Further characterisation of the opioid-mediated modulation of SON neurons has demonstrated that both  $\mu$ - and  $\kappa$ -opioid receptor agonists can alter the activity of SON neurons (Wakerley *et al.*, 1983; Brown *et al.*, 1998; Liu *et al.*, 1999). Liu and colleagues showed that DAGO inhibited glutamate-evoked EPSPs in an *in vitro* hypothalamic slice containing the SON, while it had no effect on inhibitory postsynaptic currents produced by GABA (Liu *et al.*, 1999). It also appears that  $\mu$ -opioid agonists such as morphine and the mixed  $\mu/\delta$  agonist DADLE selectively inhibit the oxytocin-containing neurons in the SON that have non-phasic activity, while the phasically active, vasopressin-containing SON neurons are generally unaffected by these  $\mu$ -opioid agonists

(Wakerley *et al.*, 1983). In contrast, the vasopressin-containing neurons are inhibited by  $\kappa$ -opioid selective ligands, as Brown and colleagues demonstrated that the  $\kappa$ -opioid receptor antagonist nor-binaltorphimine increased the activity of vasopressin neurons in the SON (Brown *et al.*, 1998). However, an inhibitory opioid influence on SON neurons may not be tonically active, as systemic naloxone had no effect on the activity of SON neurons in the unstressed state (Shibuki *et al.*, 1988; Leng *et al.*, 1992). This evidence is supported by the findings of this study, as well as previous work (Kraus *et al.*, 1996; Carr *et al.*, 1998; Gestreau *et al.*, 2000; Laorden *et al.*, 2000), where central or peripheral administration of non-selective opioid antagonists had no effect on the basal activity of SON neurons.

The evidence detailing an inhibitory influence of opioid agonists on the activity of SON neurons does not correlate well with the results of the present study, where i.c.v. naloxone decreased the number of Fos-positive cells, and hence decreased activity, in the SON of rats exposed to restraint stress. However, the fact that previous reports have shown that naloxone had no effect on SON neuronal activity suggests indirect mechanisms may attribute to the reduction in Fos-positive cells in the SON observed in the present study. Thus, another brain nucleus that contains opioid receptor populations (e.g. amygdala, LC, NTS) providing afferent input to the SON may contribute to the inhibition of these magnocellular SON neurons. One possible mechanism may be an opioid receptor-mediated disinhibition of an inhibitory GABA input to the SON (Decavel & van den Pol, 1992), which would result in a suppression of the activity SON neurons during stress. Alternatively, the presence of naloxone-insensitive receptors that have an affinity for  $\kappa$ -opioid agonists have been previously reported in the SON and PVN and may contribute to the current results (Tsushima *et al.*, 1993), with one report suggesting that these receptors may belong to the NPY family (Miura *et al.*, 1994). As a consequence, blockade of the naloxone-sensitive  $\mu$ - and  $\kappa$ -opioid receptors in the SON may unmask the potential inhibitory actions of this putative additional receptor that has an affinity for dynorphins. Obviously, further characterisation of this putative receptor and its role in the central stress response is required.

The subsequent effects on oxytocin and vasopressin release following naloxone-induced inhibition of SON neurons during restraint are unknown. With i.c.v. naloxone decreasing neuronal activity in the SON of stressed rats, plasma oxytocin concentrations would be expected to decrease. However, previous studies have shown that systemic naloxone increases oxytocin release at the level of the neurohypophysis. Therefore, measuring plasma concentrations of oxytocin in rats exposed to restraint stress that have received an i.c.v. microinjection of naloxone

would provide an interesting insight into the control of oxytocin release by the opioids. More specifically, the level of circulating oxytocin would demonstrate where opioids (and naloxone) exert the more powerful influence on oxytocin release, either at the level of the SON or posterior pituitary. The potential effect of i.c.v. naloxone on plasma vasopressin is also unclear. In contrast to physiological stressors such as i.p. hypertonic saline, psychological stressors apparently do not change plasma vasopressin levels (Sladek *et al.*, 1987; Hashimoto *et al.*, 1989b). Again, measuring plasma vasopressin would contribute to a further understanding of the physiological effects of reducing neuronal activity within the SON during psychological stress.

As naloxone is a non-selective opioid receptor antagonist, the changes in Fos production cannot be attributed to the antagonism of one specific receptor subtype, although the predominant opioid receptor subtype present in both the pPVN and SON appears to be the  $\kappa$ -opioid receptor. The  $\mu$ -opioid receptor subtype is also present in these regions, while there are very small populations of the  $\delta$  subtype (Mansour *et al.*, 1987; Mansour *et al.*, 1993; Mansour *et al.*, 1994b; Mansour *et al.*, 1994c). Central administration of more selective opioid antagonists prior to restraint stress will undoubtedly provide a much clearer picture of the association of each subtype with the central response to restraint stress, particularly in the pPVN and SON.

#### 5.4.4 CONCLUSION

In summary, rats receiving an i.c.v. microinjection of naloxone, an opioid receptor antagonist, or saline were exposed to acute restraint for 60 min. To examine the effects of naloxone on the central response to restraint stress, Fos-positive cells were visualised, counted and compared between the naloxone- and saline-treated rats in selected nuclei. In the pPVN, i.c.v. naloxone induced a significant increase in the number of Fos-positive cells and therefore an increase in the number of cells activated by restraint stress. In the SON, i.c.v. naloxone had the opposite effect, with a decrease in neuronal activation observed in this region as indicated by a significant decrease in the number of Fos-positive cells in naloxone-treated rats compared to controls. Both of these regions are involved in the control of neuroendocrine activity in the basal and stressed state. Thus, the current study presents a novel finding demonstrating that during exposure to psychological stressors such as restraint, opioids exert an inhibitory (pPVN) and stimulatory (SON) influence on regions involved in neuroendocrine and autonomic regulation. Further studies that extend this investigation, such as measuring neuroendocrine changes in i.c.v.

naloxone-treated rats exposed to restraint stress, will provide an interesting insight into the modulation of the central response to psychological stressors by the opioid system.

# GENERAL DISCUSSION



Discuss  
**dis-kus'**

Verb - to examine by  
argument; sift the  
considerations for and  
against; debate; talk  
over.

## **CHAPTER 6**

### **GENERAL DISCUSSION**

Neuropeptides are widely distributed throughout the CNS, and as they are involved in the modulation of neurotransmission in many nuclei implicated in the regulation of autonomic processing, they have the capacity to modulate many physiological processes. The present thesis targetted 3 neuropeptide systems and their neurochemical markers; namely, the opioid, GAL and NPY systems. The opioid system contains many neuropeptides, with some of their fragments also exhibiting significant biological activity, and the present thesis studied the expression of 2 opioid peptide precursors, prepro-ENK and proDYN, in addition to the distribution and density of the 3 main opioid receptor subtypes,  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors. To investigate the GAL system, the expression of its precursor, prepro-GAL, and the density of [<sup>125</sup>I]-GAL binding sites were quantified at selected levels of the rat CNS. Finally, levels of prepro-NPY mRNA, the precursor for NPY, were measured as an indicator of activity of the central NPY system.

These 3 neuropeptide systems have been the focus of a great deal of research in their own rights since their respective discoveries over 2 decades ago. As outlined in detail in section 1.3 and in the pertinent parts of each chapter discussion, the opioids, GAL and NPY have been associated with functions such as cardiovascular regulation, analgesia, central control of feeding, modulation of neuroendocrine outputs and the patterning of the central stress response. Furthermore, comparison of the activity and function of each of these neuropeptide systems in the CNS of SHR, a commonly used rodent model of genetic hypertension, with normotensive WKY controls has revealed a number of differences, some of which may contribute to the hypertensive state (see section 1.4.2).

The present thesis therefore aimed to further characterise these neuropeptide systems in the CNS of normotensive WKY and hypertensive SHR. From this platform, the function of the opioid, GAL and NPY systems were monitored and quantified in rats exposed to a 10 day restraint paradigm and the response of the opioid, GAL and NPY systems to acute and chronic restraint was compared between WKY and SHR. These studies therefore contribute to the current understanding of how the opioid, GAL and NPY systems function in unstressed and

stressed WKY and in SHR rats that are hypertensive, hypoalgesic and hyperactive compared to WKY (see section 1.4.3).

## 6.1 NEUROCHEMICAL DIFFERENCES BETWEEN WKY AND SHR

In chapter 3, the levels of expression of prepro-ENK, proDYN, prepro-GAL and prepro-NPY mRNA were compared throughout the CNS of WKY and SHR using a standard ISHH protocol. The use of oligonucleotide probes targetted at a portion of the sequence of each neuropeptide precursor permitted visualisation of a topographic distribution profile that was specific and distinct for prepro-ENK, proDYN, prepro-GAL and prepro-NPY. Following statistical comparison, significantly altered expression of each of these neuropeptide precursors were detected in discrete nuclei in SHR when compared to WKY. These significant changes were specific for each neuropeptide and detected in a variety of regions in the forebrain, pons and medulla oblongata of SHR.

In the medulla oblongata, the NTS, CVLM and RVLM are the main components of the sympathetic branch of the baroreceptor reflex loop that regulates BP and HR in the rat during rest and in stressful situations (see Dampney, 1994). In the NTS, proDYN mRNA levels were found to be significantly reduced in SHR compared to WKY. Furthermore, prepro-ENK and prepro-GAL mRNA expression were significantly increased in the RVLM of SHR when compared to the normotensive WKY controls, while a recent study detected prepro-ENK mRNA in the non-C1 vasomotor spinally-projecting neurons of the RVLM (Guyenet *et al.*, 2001). Thus, changes in the expression of proDYN, prepro-ENK and prepro-GAL mRNA in the NTS and RVLM may be contributing to the increased BP status and/or sympathetic outflow previously observed in SHR (Judy *et al.*, 1976; Judy *et al.*, 1979; Folkow, 1982).

As described previously, there are many more phenotypic differences between WKY and SHR other than hypertension, with SHR reported to have elevated HPA axis activity, altered plasma hormone levels, hypoalgesia and hyperactivity when compared to WKY (see section 1.4). Consequently, the alterations in levels of prepro-ENK, proDYN, prepro-GAL and prepro-NPY mRNA that were detected in the CNS of SHR when compared to WKY may be contributing to one or all of these phenotypic differences. For example, proDYN mRNA and prepro-GAL mRNA levels were significantly reduced in the SON of SHR compared to WKY.

Dynorphin and GAL are colocalised with vasopressin and oxytocin in neurons of the SON (Molineaux *et al.*, 1984; Skofitsch *et al.*, 1989; Meister *et al.*, 1990b), and have the capacity to regulate the release of these hormones (see section 3.4). Thus, considering that SHR have been reported to have altered expression of vasopressin and oxytocin mRNA in the SON, in addition to differences in the plasma levels of vasopressin and oxytocin (Morris *et al.*, 1983; Rosella-Dampman *et al.*, 1985; Van Tol *et al.*, 1988), it is possible that the changes in proDYN and prepro-GAL mRNA expression observed in chapter 3 may be contributing to these hormonal variations in SHR.

Chapter 4 documented additional studies of the opioid and GAL systems in the CNS of WKY and SHR, but rather than studying gene expression, the density of  $\mu$ - ( $[^{125}\text{I}]\text{-FK 33-824}$ ),  $\delta$ - ( $[^3\text{H}]\text{-naltrindole}$ ) and  $\kappa$ -opioid ( $[^3\text{H}]\text{-U69-593}$ ) and GAL ( $[^{125}\text{I}]\text{-GAL}$ ) receptors were quantified and compared between WKY and SHR. Autoradiographic comparison of the density of each of the opioid receptor subtypes ( $\mu$ ,  $\delta$  and  $\kappa$ ) in the CNS of WKY and SHR revealed that significant differences exist at all levels of the neuraxis in SHR compared to WKY. Moreover, these changes in  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptor density were ligand- and region-specific. Similar to the changes in prepro-ENK and proDYN mRNA expression reported in chapter 3, differences between strains in opioid receptor density were localised in regions involved in a variety of physiological functions. SHR are reported to be hypoalgesic when compared to WKY (Sitsen & de Jong, 1983), and many central regions may be contributing to this strain difference. It was demonstrated in chapter 4 that  $[^{125}\text{I}]\text{-FK 33-824}$  binding site density was altered in the thalamus, cortex, BL and cerebellum, while the density of  $[^3\text{H}]\text{-naltrindole}$  binding sites were reduced in the CPu. In addition, the Me was found to contain a significantly elevated density of  $[^3\text{H}]\text{-U69-593}$  binding sites. Previous studies have demonstrated that opioids can modulate nociception within each of these regions (see section 4.4), and the finding that opioid receptor density was altered in these regions in SHR when compared to WKY suggests that the opioid system may be associated with the atypical regulation of nociception in one, some or all of these nuclei in SHR.

Changes in opioid receptor density were also located in SHR in central regions implicated in the regulation of the cardiovascular system. Examples of regions exhibiting altered opioid receptor levels in SHR compared to WKY include the NTS ( $\delta$ ,  $\kappa$ ) and CVLM ( $\delta$ ), regions considered to be integral components of the central baroreceptor reflex loop (Dampney, 1994). These results therefore provide further evidence that altered levels of opioid receptors are localised in SHR in regions important for the regulation of various processes, such as

cardiovascular control and nociception, and may contribute to the altered function of these processes previously observed in this hypertensive strain.

In the majority of regions where [<sup>3</sup>H]-naltrindole binding site density was assessed, SHR were found to contain a significantly lower level of [<sup>3</sup>H]-naltrindole binding sites than WKY. In addition, SHR rats contained a significantly elevated density of [<sup>3</sup>H]-U69-593 binding sites in many central nuclei when compared to WKY. Some of these differences were quite large, and warrant further investigation. A set of experiments that measures the  $K_d$  and  $B_{MAX}$  for  $\delta$ - and  $\kappa$ -opioid receptors in a variety of discrete central nuclei would provide further information regarding the underlying cause of the results described in chapter 4. It is possible that in SHR, the  $\delta$ - and/or  $\kappa$ -opioid receptor may have an altered affinity for selective opioid-receptor ligands or differences in maximum receptor number or both. The existence of opioid receptor hetero- and homo-dimers has been reported (Cvejic & Devi, 1997; Jordan & Devi, 1999), and a shift in the balance of these opioid receptor complexes may contribute to alterations in  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptor density in SHR. Furthermore, there may also be differences in terms of coupling with second messenger systems and function of the  $\delta$ - and  $\kappa$ -opioid receptor in SHR that could be addressed in future experiments.

The experiments outlined in chapters 3 and 4 allow parallels to be drawn between changes in precursor expression and receptor density. In the NTS, prepro-ENK mRNA levels were not significantly altered in SHR, while a significantly reduced level of proDYN mRNA expression was detected in SHR when compared to WKY. As described in section 1.3.1.1, enzymatic processing of proDYN generates not only the dynorphins that bind to the  $\kappa$ -opioid receptor, but also multiple copies of Leu-enkephalin which can bind to  $\mu$ - and  $\delta$ -opioid receptors (Raynor *et al.*, 1994). Thus, changes in proDYN mRNA in the NTS may produce or be associated with changes in opioid receptor density in the NTS, if proDYN mRNA is present in interneurons, or in regions receiving efferent projections from the NTS that contain dynorphin. In the NTS of SHR,  $\delta$ -opioid receptor density was found to be decreased, while the density of  $\kappa$ -opioid receptors was elevated when compared to WKY. In addition,  $\delta$ -opioid receptor density was reduced in the CVLM, with [<sup>3</sup>H]-U69-593 binding sites elevated in the PVN, two regions known to receive afferent input from the NTS (Swanson & Sawchenko, 1983; Van Giersbergen *et al.*, 1992). The relationship between the NTS and CVLM/PVN in terms of altered opioid neurochemistry may be an important influence on the status of the cardiovascular and/or neuroendocrine systems in SHR, and as such provide a target for future studies.

Another region where a correlation between changes in prepro-ENK mRNA expression and opioid receptor density may exist is the cerebellum, where both prepro-ENK mRNA levels and the density of  $\mu$ -opioid receptors were elevated in SHR compared to WKY. The cerebellum has been implicated in cardiovascular control, movement and nociception (Dey & Ray, 1982; Cicirata *et al.*, 1989; Talman *et al.*, 1991), and the changes in opioid neurochemistry in this region may contribute to the phenotypic differences observed in SHR that are related to these functions.

These data therefore provide future targets for research directed at finding underlying neurochemical alterations that may contribute to the phenotype characteristic of the SHR strain, such as hypertension, hypoalgesia and hyperactivity as compared to WKY (see section 1.4.3). Similar experiments that investigate the neurochemistry of markers of the opioid, GAL, NPY and other modulatory systems could be completed using normotensive rats other than WKY and additional rodent models of hypertension such as the Milan and Genetically hypertensive strains (Dominiczak & Lindpaintner, 1994). Comparison of the results of these additional studies with the present thesis would provide information regarding the nature of the neurochemical changes outlined in chapters 3 and 4. Specifically, observation of similar changes in SHR and other hypertensive models would suggest that changes in expression of neuropeptide precursors or receptor density in a particular region may be contributing to the hypertensive state. In contrast, changes in gene expression or receptor density in the opioid, GAL or NPY systems that vary between strains, irrespective of the resting level of BP, may be a strain-related difference that could be attributed to the activity of another physiological function, such as behaviour or neuroendocrine control.

## 6.2 RESTRAINT AND THE OPIOID, GAL AND NPY SYSTEMS

The restraint paradigm used throughout the thesis consisted of placing the rat in a perspex tube for 60 min for 1 to 10 consecutive days, after which the rat was decapitated, its brain sectioned and processed for expression of mRNA encoding neuropeptide precursors (chapter 3), density of opioid and GAL receptors (chapter 4) or Fos immunohistochemistry (chapter 5). This restraint paradigm was shown to produce changes in HR and MAP in chapter 2 (section 2.4), and previously published studies have demonstrated the cardiovascular, neuroendocrine, thermic and

nociceptive effects of restraint (Chen & Herbert, 1995; Gamaro *et al.*, 1998; Stamp & Herbert, 1999; McDougall *et al.*, 2000).

As outlined in Chapter 3, the levels of expression of prepro-ENK, proDYN, prepro-GAL and prepro-NPY mRNA were measured in the CNS of rats exposed to 0, 1, 3, 5 or 10 consecutive days of the restraint paradigm. Changes in the levels of the transcripts encoding these neuropeptides were detected across all levels of the WKY CNS that were analysed, and these alterations were neuropeptide- and region-specific and differed according to the number of days of restraint. Acute (1 session) restraint induced changes in prepro-ENK mRNA in the WKY CPu, Ce and cerebellum, while proDYN mRNA levels were altered in regions such as the VMH, DG and Ce after acute restraint. Prepro-GAL mRNA expression was found to be significantly altered in WKY in the Ce following exposure to 1 day of the restraint paradigm, while acute restraint also produced changes in prepro-NPY mRNA in the ARC of WKY (see section 3.3). These data demonstrate that the expression of precursor mRNA, which is likely to be associated with an increased synthesis of the pertinent neuropeptide, is dynamically regulated and can be altered to address each environmental situation when required.

Repeated exposure to the restraint paradigm also elicited changes in gene expression, with 3 sessions of restraint inducing a significant increase in prepro-ENK mRNA levels in the WKY LC, while 5 periods of restraint produced a significantly altered expression of prepro-ENK mRNA in the Pir, NTS and RVLM of WKY. Chronic restraint produced significant changes in proDYN mRNA levels in the VMH and DG, while no changes in prepro-GAL mRNA levels were observed in any region of the WKY CNS after repeated restraint. In addition, prepro-NPY mRNA levels in the cortex of WKY were significantly reduced after 5 days of the restraint paradigm. Some of the changes outlined above occurred in regions where acute restraint did not produce any alterations in gene expression (e.g. RVLM – prepro-ENK), demonstrating that the length of the stressor, in this case the restraint paradigm, influences the central neural response and recruitment of individual regions.

Furthermore, there were some regions that were activated by an acute session of restraint that were not sensitive to repeated restraint. Examples of such regions include the PeF (prepro-ENK), Ce (proDYN and prepro-GAL) and ARC (prepro-NPY). In these same regions, following the initial response, continued exposure to the restraint paradigm produced a graded return towards basal levels of expression. This type of temporal response pattern clearly demonstrates the plasticity of central neurons during repeated stimulation (i.e. restraint). Neuronal plasticity does not appear to be confined to a particular phenotype of neuron, with a

similar response profile to repeated restraint observed for each neuropeptide in a region-specific manner. Moreover, the alteration in expression of the mRNA encoding each neuropeptide precursor during the 10 day restraint paradigm is an indication of the level of adaptation occurring in the CNS, with adaptation presumably occurring in the neurons providing afferent input to the specific nucleus. Adaptation to chronic restraint has also been measured in many physiological systems, such as the HPA axis and sympathoadrenal system (see section 1.2.1). It is possible that one of the many temporal responses to 10 sessions of restraint described in chapter 3 may be associated with the activity of a physiological system, and further studies that directly correlate changes in gene expression with changes in plasma hormone levels, HR, BP or behaviour during repeated restraint could provide more information on this area.

Chapter 4 documented the effects of 0, 1, 3, 5 or 10 days of exposure to the restraint paradigm on receptor density in selected regions of the WKY CNS. Acute restraint elicited significant changes in the density of opioid ( $\mu$ ,  $\delta$  and  $\kappa$ ) and GAL receptors in various nuclei. In the cortex of WKY, [ $^{125}$ I]-FK 33-824 ( $\mu$ ) binding site density was decreased by acute restraint, while 1 period of restraint increase the density of [ $^3$ H]-naltrindole ( $\delta$ ) binding sites in the VMH and BM. In WKY, [ $^{125}$ I]-GAL binding site density in the Ce and Me was significantly reduced by 1 period of restraint. Chronic exposure to the restraint paradigm also produced significant changes in receptor density, with  $\mu$ -opioid receptor density in the PB of WKY increasing after 10 sessions of restraint. In addition, 3, 5 and 10 days of the restraint paradigm induced significant increases in [ $^3$ H]-naltrindole binding sites in the VMH of WKY, and significant changes in [ $^3$ H]-U69-593 ( $\kappa$ ) binding site density were observed in the Par (5 sessions) and medial NTS (3 sessions). Exposure to the restraint paradigm for 3 and 5 days also elicited a reduction in [ $^{125}$ I]-GAL binding site density in the Ce and VMH of WKY. Thus, it can be seen that both acute and chronic restraint induced significant changes in receptor density throughout the WKY neuraxis.

Furthermore, closer analysis of the temporal response to the 10 day restraint paradigm revealed the presence of 4 distinct response patterns. Firstly, some regions responded to acute restraint, but repeated exposure to the restraint paradigm resulted in a return to levels of receptor density comparable to unstressed rats. Some examples include the cortex ( $\mu$ ) and Me ([ $^{125}$ I]-GAL), with regions such as the Ce ([ $^{125}$ I]-GAL) and VMH ([ $^{125}$ I]-GAL) exhibiting a slower return to basal levels of receptor density. Another group of regions were sensitive to chronic, but not acute, periods of restraint and include the PB ( $\mu$ ), cortex ( $\delta$ ) and medial NTS ( $\kappa$ ). As described earlier, these temporal response patterns demonstrate the degree of plasticity present within the CNS in response to repeated restraint.

The third response pattern was a change in receptor density that was maintained throughout the entire restraint paradigm, and this type of response was detected in the VMH ( $\delta$ ). This response profile may indicate that the region is involved in a particular component of the stress response that is active irrespective of the length or duration of the stressor. Alternatively, the particular region may alter its response to chronic restraint on a time scale that extends for a period longer than 10 days. Further experiments are obviously required to ascertain the reasons for a response profile such as this, and may involve (i) restraint periods that last longer than 10 days, (ii) restraint sessions that continue for more than 60 min and (iii) exposure to other stressors to investigate the stressor-specificity of the response.

Another response pattern that was evident in the data reported in both chapter 3 and 4 was an insensitivity to the restraint paradigm, and this was exhibited by regions such as the thalamus ( $\mu$ ), PVA ( $\kappa$ ), NTS ( $[^{125}\text{I}]\text{-GAL}$ ) and CVLM ( $\delta$ ). These data demonstrate that the central response to restraint does not involve every neuronal phenotype in each individual nucleus. It is also possible that these regions respond to restraint, but the change is not detectable or occurs within the first few minutes of restraint and returns to resting levels during the 60 min restraint session. This type of response has been observed by previous studies, with restraint producing significant changes in prepro-ENK mRNA expression after 2 min (Boone & McMillen, 1994a). Moreover, physiological responses, such as hypertension and tachycardia, are also detected immediately after the initiation of restraint (McDougall *et al.*, 2000). It is therefore possible that the 60 min restraint paradigm may underestimate the changes in gene expression and receptor density that were reported in chapters 3 and 4, as an initial change that may occur during the first 5-10 min of restraint may be returning towards baseline levels after 60 min. However, the presence of such a response would need to be investigated using different periods of restraint, ranging from 5 min to 180 min. This would provide an indication of the degree of neuronal plasticity during continuous stimulation or exposure to a psychological stressor such as restraint. Moreover, if the level of gene expression or receptor density over this time scale was combined with a repeated physiological measure, such as plasma hormone concentration, HR or BP, a more accurate association between the activation of central nuclei and the function of physiological systems during stress could be gained.

### 6.2.1 THE NEURAL RESPONSE TO RESTRAINT IN WKY AND SHR

The atypical stress response observed in the hypertensive SHR has been well documented (e.g. Narváez *et al.*, 1993; McDougall *et al.*, 2000). In chapters 3 and 4, I sought to investigate the response of the opioid, GAL and NPY systems in SHR to a 10 day restraint paradigm and how the temporal response patterns compared to those of WKY, a normotensive control strain. Changes in expression of the transcripts encoding prepro-ENK, proDYN, prepro-GAL and prepro-NPY, as well as changes in the density of opioid ( $\mu$ ,  $\delta$  and  $\kappa$ ) and GAL receptors were detected following exposure to acute and chronic restraint in a number of central regions. Acute restraint induced significant changes in gene expression in SHR in regions such as the SON (proDYN), Ce (proDYN), LC (prepro-GAL), cerebellum (prepro-ENK) and VLM (prepro-NPY). Furthermore, when compared to unstressed controls, receptor density was significantly altered in regions such as the PVN ( $\kappa$ ), Me ( $\kappa$ ), BL ( $\mu$ ) and NTS ( $\delta$ ) following 1 period of restraint stress, demonstrating that acute exposure to this stressor can elicit neurochemical changes in the CNS of SHR.

Significant alterations in the levels of expression for the 4 neuropeptide precursors were also detected in multiple regions of SHR after repeated exposure to the restraint paradigm compared to unstressed controls, including the SON (proDYN - 3, 5 and 10 sessions; prepro-GAL - 10 sessions), Ce (proDYN and prepro-GAL - 3, 5 and 10 sessions), LC (prepro-ENK - 3 sessions; prepro-NPY - 5 sessions), RVLM (prepro-ENK - 5 sessions) and NTS (proDYN - 3, 5 and 10 sessions). In addition, chronic restraint elicited significant changes in receptor density in SHR in regions such as the PVN ( $\kappa$  - 3 and 5 sessions), Me ( $\kappa$  - 3, 5 and 10 sessions), BL ( $\mu$  - 3 and 10 sessions), lateral PB ( $[^{125}\text{I}]\text{-GAL}$  - 5 sessions), NTS ( $\delta$  - 3, 5 and 10 days;  $\kappa$  - 5 sessions) and CVLM ( $\delta$ ; 5 sessions). These data demonstrate that the neural response to repeated restraint is functional within the CNS of SHR.

Analysis of the temporal response to repeated restraint revealed that many of the patterns observed in WKY in the previous section were also evident in SHR. There were regions that responded only to acute restraint, and these included the Pir ( $[^{125}\text{I}]\text{-GAL}$ ), LC (prepro-GAL) and VLM (prepro-NPY). Moreover, in these regions, it was apparent that mRNA levels or receptor density was returning towards baseline values, albeit slowly in some cases. Data such as these demonstrate that SHR have the capacity to adapt to restraint stress. In other regions, chronic restraint alone produced changes in gene expression or receptor density, and these included the SON (prepro-GAL), commissural NTS ( $\kappa$ ), CVLM ( $\delta$ ) and RVLM (prepro-ENK). A number of

central regions in SHR exhibited a sustained response to restraint that persisted for the majority, if not all, of the 10 days of the paradigm. Examples of these regions include the SON (proDYN), Ce (proDYN and prepro-GAL), Me ( $\kappa$ ), cerebellum (prepro-ENK) and NTS (proDYN and  $\delta$ ). In addition, there were regions in SHR that were not perturbed by the restraint paradigm, although it is possible that in these particular nuclei, restraint produced changes in transcript or receptor levels for a neurotransmitter/neuropeptide that was not measured in the present thesis. The PVN (prepro-ENK and prepro-GAL), PeF (prepro-ENK), BM ( $\mu$  and  $\delta$ ), Ce ( $[^{125}\text{I}]\text{-GAL}$ ), Me ( $[^{125}\text{I}]\text{-GAL}$ ) and NTS (prepro-GAL, prepro-NPY,  $\mu$  and  $[^{125}\text{I}]\text{-GAL}$ ) in SHR were regions that did not exhibit alterations following restraint stress in the respective parameters.

A response pattern in SHR that was not observed in WKY was a biphasic profile, where an initial increase in gene expression or receptor density was immediately followed by a significant decrease. Regions in SHR where this interesting response profile occurred were the commissural and medial NTS when measuring the changes in  $[^3\text{H}]\text{-naltrindole}$  binding sites induced by the 10 day restraint paradigm (see Figure 4.8G and 4.8H). Acute (1 period) restraint produced a significant increase in the density of  $\delta$ -opioid receptors in the SHR NTS, with 3 sessions inducing a significant reduction in  $[^3\text{H}]\text{-naltrindole}$  binding site density followed by a gradual return towards basal levels after exposure to 5 and 10 days of the restraint paradigm. Although the significant changes observed after 1 period and 3/5 periods of restraint are opposite in direction, they may (i) reflect shifts in active states of the receptor not delineated by  $[^3\text{H}]\text{-naltrindole}$  or (ii) concentration fluctuations in complexes of the  $\delta$ -opioid receptor that alter the affinity of the  $\delta$ -opioid receptor for  $[^3\text{H}]\text{-naltrindole}$ . However, no changes in  $[^3\text{H}]\text{-naltrindole}$  binding site density were observed in the NTS of WKY at any stage during the restraint paradigm, suggesting that this type of response profile is restricted to SHR. Further studies that associate functional changes in specific physiological systems, such as the cardiovascular or neuroendocrine systems, during exposure to stress with  $\delta$ -opioid receptor density in the NTS are required to determine the significance and underlying causes of this response profile.

A second biphasic temporal response to the restraint paradigm was documented in chapter 3 in SHR in regions such as the VMH (prepro-ENK and proDYN), CPu (prepro-ENK and proDYN) DG (proDYN and prepro-NPY) and ARC (prepro-NPY). In these regions, acute (1 session) restraint produced an increase in expression of the specific neuropeptide mRNA. Exposure to 3 and 10 days of the restraint paradigm also produced a significant increase in neuropeptide mRNA expression that had a similar magnitude. However, 5 periods of restraint produced either a very small increase in neuropeptide mRNA expression or no change at all in

these particular regions when compared to basal levels of expression. Thus, it appears that these specific neuropeptide-containing neurons are activated by acute restraint, and the expression of these genes is elevated for at least 3 days of the restraint paradigm. After day 3, the expression of prepro-ENK, proDYN and prepro-NPY mRNA returns towards baseline levels. As the restraint paradigm continues, at some point prior to day 10 there is a signal for the expression of these neuropeptide precursors to increase for the second time, with this secondary increase evident on day 10 of the restraint paradigm. As there was a relatively large time period between day 5 and day 10 of the restraint paradigm, the exact time when the secondary elevation in gene expression occurs is unclear. Additional studies using the same restraint paradigm, but with measurements of gene expression completed at more time points between days 5 and 10, would provide more information regarding the exact time course of restraint-induced changes in prepro-ENK, proDYN and prepro-NPY mRNA expression during repeated restraint. These studies are, however, logistically complicated. Further studies are also required to determine the biological significance of this particular biphasic response, where specific subsets of neurons are activated on 2 separate occasions during a 10 day restraint paradigm. A biphasic cardiovascular response was not observed in SHR following a similar restraint paradigm, while the restraint-induced release of corticosterone in normotensive rats is attenuated by repeated exposure (Chen & Herbert, 1995; McDougall *et al.*, 2000). Therefore, the activity of additional physiological systems in SHR rats exposed to repeated restraint requires careful monitoring to determine whether a dual response exists and how this may correlate with the biphasic temporal response profiles observed in SHR in specific brain regions as outlined in chapter 3.

A 10 day biphasic response profile such as that described above demonstrates that the neural network governing the stress response is plastic, and still retains its sensitivity during repeated exposure to restraint. However, this particular biphasic temporal response was not observed in any central nucleus in WKY, and furthermore, neurons containing prepro-GAL mRNA in the SHR CNS did not exhibit this type of response pattern, demonstrating that the response profile is strain- and neuropeptide-specific. Additional studies that quantify prepro-ENK, proDYN and prepro-NPY mRNA in regions such as the VMH, DG and CPu in rats with experimentally-induced hypertension (e.g. DOCA salt-treated rats) that are also exposed to 10 days of restraint would assist in determining whether this biphasic temporal response profile is associated with hypertension.

The biphasic temporal response profiles discussed above provide evidence of regions where a marked contrast between WKY and SHR regarding restraint-induced changes in gene expression

or receptor density was observed. As outlined in chapter 3, examples of regions that responded differently in WKY and SHR to the restraint paradigm included the PeF (prepro-ENK), SON (proDYN and prepro-GAL), ARC (prepro-NPY), Ce (proDYN and prepro-GAL) and LC (prepro-ENK). Chapter 4 also reported changes in receptor density induced by the restraint paradigm that were significantly different in SHR compared to WKY in regions such as the BL ( $\mu$ ), Ce ( $[^{125}\text{I}]\text{-GAL}$ ), PB ( $\mu$ ), LC ( $\delta$ ) and NTS ( $\delta$ ).

In some of these regions, restraint only produced significant effects in WKY (e.g. PeF (prepro-ENK), Ce ( $[^{125}\text{I}]\text{-GAL}$ ), PB ( $\mu$ )). In other discrete nuclei, significant changes in gene expression or receptor density were evident only in SHR, including regions such as the SON (prepro-GAL) and NTS ( $\delta$ ). Furthermore, while restraint elicited significant changes in gene expression or receptor density in both strains in a particular region, the time course or magnitude of these changes were sometimes significantly different between strains. Regions where acute restraint produced significant effects in WKY but not SHR, and repeated restraint induced changes in SHR but not WKY included the Ce (prepro-GAL and proDYN) and cortex ( $\mu$ ). In addition, there were regions in both strains that responded in a similar fashion to the restraint paradigm, but the magnitude of the changes were greater in SHR than WKY, and this was observed in regions such as the Me ( $\kappa$ ) and cerebellum (prepro-ENK). It is therefore apparent that the present restraint paradigm produced a unique topographic profile of significant alterations in both gene expression and receptor density in WKY and SHR.

As outlined in the appropriate discussion sections in chapters 3 and 4, the significantly altered temporal response profiles between WKY and SHR may be associated with the atypical physiological response to stress previously observed in SHR. Stressors such as restraint, immobilisation and forced swimming have produced altered responses in the cardiovascular system, HPA axis and stress-induced hyperglycaemia in SHR when compared to WKY (Sowers *et al.*, 1981; Armario *et al.*, 1995; Imaki *et al.*, 1998; McDougall *et al.*, 2000). Furthermore, it is possible that phenotypical variations in SHR compared to WKY, including hypertension, hypoalgesia, altered hormone release and hyperactivity (see section 1.4.3) may also contribute to the significantly altered neural response to restraint observed in chapters 3 and 4. To further strengthen any associations between an altered stress response and a trait characteristic of SHR, similar experiments need to be conducted using additional rat strains. For example, an experimental rodent model of hypertension (such as the DOCA salt-treated rats) could be used to determine whether similar changes in central neurochemistry exist in DOCA salt-treated rats and SHR, and if so, then hypertension may be an underlying causal factor. Another approach is to

use SHR that have been experimentally treated so that they have a lower resting MAP compared to "normal" SHR. This can be achieved using the chronic administration of drugs such as naloxone and captopril in young SHR (Quock *et al.*, 1984; Berecek *et al.*, 1987). Comparison of the neurochemistry of these treated SHR with "normal" SHR and WKY may provide some insight into the association between cardiovascular changes and the activity of neurons of a particular phenotype during stress. In addition, the use of extra normotensive control strains, such as the SD, Wistar and Long Evans may also provide different perspectives on the data. Studies by Armario and colleagues and Gómez and co-workers compared the behavioural and physiological response to a swimming stress, and found that in some cases, SHR exhibited similar responses to other normotensive strains such as the Brown-Norway and Fischer strains (Armario *et al.*, 1995; Gómez *et al.*, 1996). Thus, it appears that the response to stress is strain-specific, and the choice of a control strain can have a significant bearing on the results.

In recent times, selective inbreeding of WKY have produced substrains that are hyperreactive and normotensive, and rats that are hypertensive but not hyperreactive (Hendley *et al.*, 1991). Using these strains in conjunction with SHR and WKY, Hendley and colleagues found that the hyperactive/normotensive WKY, and not normoreactive/hypertensive WKY, exhibited the hypersensitive catecholamine response to footshock characteristic of SHR (Hendley *et al.*, 1988). Selective breeding such as this provides researchers with valuable rodent models that assist in associating neurochemical changes with the phenotypic traits of a specific strain, both during exposure to stress and in the basal state. Therefore, completion of similar experiments as outlined in chapters 3 and 4 using these selectively bred substrains of SHR will extend the findings of the present thesis, and provide researchers with a greater understanding of the main physiological contributors to the atypical stress response in SHR.

### 6.3 I.C.V. NALOXONE AND RESTRAINT-INDUCED CHANGES IN FOS IMMUNOHISTOCHEMISTRY

The studies presented in chapters 3 and 4 represent initial steps in determining the importance of particular neural circuits to the central stress response and the involvement of specific neuropeptides. Chapters 3 and 4 documented that in the rat CNS, neurons of various phenotypes can respond to acute restraint, and that repeated exposure to the same stressor could produce a

modified response. This neuronal adaptation clearly demonstrates the plasticity of the CNS, where various levels of stimulation can lead to quite different neural responses. However, it is not known whether the dynamic changes outlined in chapters 3 and 4 are primarily occurring to mediate or produce alterations in the activity of a particular component of the physiological stress response, or if the significant changes in a particular nucleus are a side effect of altered neuronal activity within the CNS during the central stress response.

Similarly, studies measuring the expression of Fos protein in discrete nuclei in the CNS of rats exposed to one session of restraint do not identify regions of primary importance to a specific component of the central stress response. Chapter 5 described how the present restraint paradigm has the capacity to elicit an increase in Fos production in many central regions, including those where restraint-induced alterations in receptor density and gene expression were observed in chapters 3 and 4. Examples include the Me, SON, LC and NTS. However, the Ce was one region where restraint did not increase the neuronal expression of Fos protein. In contrast to this finding, chapters 3 and 4 reported that acute (1 session) restraint increased prepro-ENK, proDYN and prepro-GAL mRNA and decreased [<sup>125</sup>I]-GAL binding site density in the Ce of WKY. It is therefore apparent that restraint is altering neuronal activity in the Ce, but this is not being translated into altered Fos-ir in the same region. Reasons for this discrepancy were outlined in chapter 5 (section 5.4.2), and could be attributed to neurons using an immediate early gene other than *c-fos*, a net inhibition of Ce neurons through an increase in GABA-mediated modulation or a different time course of neuronal activation that is shorter than 120min, which was the injection/perfusion interval used in chapter 5. An immunohistochemical study that looks at the colocalisation of Leu- or Met-enkephalin-ir, dynorphin A-ir or GAL-ir with Fos protein in the Ce of rats exposed to restraint would provide further data regarding the activity of a particular phenotype of Ce neurons during restraint. At the same time, an investigation of the colocalisation of GAL, the enkephalins, dynorphin A, CRF and GABA in the neurons of the Ce would also provide information regarding the role of GAL and the opioids in various processes regulated by the Ce, such as the stress-induced pressor response (Wu *et al.*, 1999).

The experiments in chapter 5 were undertaken to extend the results described in chapters 3 and 4 and to investigate the biological significance of the restraint-induced changes in the opioid system. To achieve this, naloxone or saline was centrally administered (i.c.v.) and the changes in neuronal activation in selected CNS nuclei was quantified in rats exposed to acute restraint. Central administration of 100nmol naloxone in unstressed rats did not induce any significant

change in Fos production in any of the regions under investigation, which included the Ce, RVLM and NTS, when compared with rats receiving i.c.v. saline. Moreover, this same dose of naloxone had no significant effect on MAP. In rats exposed to one session of restraint, prior i.c.v. microinjection of 100nmol naloxone had no significant effect on Fos-ir levels in many regions, such as the Me, Ce, LC, NTS and RVLM. However, i.c.v. naloxone pre-treatment did induce significant changes in neuronal activity in the hypothalamic PVN and SON of rats exposed to 1 session of restraint.

In the SON, the number of Fos-positive cells were significantly decreased in restrained rats receiving i.c.v. naloxone when compared to saline-treated rats that were exposed to the restraint paradigm. This finding suggests that the release of an opioid peptide in the vicinity of the SON during restraint is stimulating the neurons of the SON. Chapter 3 reported that 1 session of restraint did not produce any significant changes in proDYN or prepro-GAL mRNA expression in WKY, while other studies have reported changes in oxytocin, CRF and NOS mRNA following restraint (Luo *et al.*, 1994; Miyata *et al.*, 1995; Krukoff & Khalili, 1997). Furthermore, vasopressin mRNA expression was not altered in the SON after exposure to stressors such as restraint and i.p. hypertonic saline (Lightman & Young, 1987; Harbuz *et al.*, 1994; Herman, 1995), demonstrating that stress-induced changes in SON neurons are neuropeptide-specific.

As discussed in chapter 5, the  $\kappa$ -opioid receptor is the prominent opioid receptor subtype found in the SON, although  $\mu$ -opioid receptor agonists such as DAGO can also inhibit the activity of oxytocin-containing neurons in the SON (Wakerley *et al.*, 1983). Interestingly,  $\kappa$ -opioid receptor antagonists stimulate vasopressin neurons within the SON, but not neurons containing oxytocin (Wakerley *et al.*, 1983). Thus,  $\mu$ - and  $\kappa$ -opioid receptors appear to have different roles in the SON. The dose of naloxone used in chapter 5 does not differentiate between the  $\mu$ - and  $\kappa$ -opioid receptor, demonstrating a requirement for further studies to determine the functional effects resulting from opioid receptor blockade. Microinjection (i.c.v.) of antagonists selective for the  $\mu$ -opioid receptor such as CTOP or  $\kappa$ -opioid receptor such as nor-binaltorphimine and subsequent quantification of Fos-positive cells in the SON (and other central regions) of rats exposed to restraint would demonstrate which opioid receptor subtype is being antagonised by naloxone in the present study. Previous evidence, where naloxone did not inhibit the effects of dynorphin within the SON (Tsushima *et al.*, 1993), suggest that the  $\mu$ -opioid receptor may be the main target of naloxone in the SON. Furthermore, correlation of changes in

Fos-ir with plasma levels of vasopressin and/or oxytocin during restraint in future experiments should also provide an insight into the functional effects of opioid receptor blockade in the SON.

In the PVN of acutely restrained rats, i.c.v. naloxone increased the expression of Fos-ir when compared to saline-treated WKY exposed to the same restraint paradigm. Moreover, this increase in neuronal activity induced by i.c.v. naloxone was limited to the pPVN, a subregion of the PVN that projects to the median eminence, medulla oblongata (RVLM, NTS) and spinal cord (Swanson & Kuypers, 1980; Swanson *et al.*, 1983; Shafton *et al.*, 1998; Pyner & Coote, 2000). It therefore appears that an endogenously released opioid is inhibiting the neurons of the pPVN during exposure to restraint. Moreover, this inhibition is not tonically active, as i.c.v. naloxone administration alone did not alter Fos expression in the pPVN of unrestrained control rats. The pPVN receives afferent input from neurons containing many opioid peptides, such as the enkephalins, dynorphins and  $\beta$ -endorphin (see chapter 5), suggesting that naloxone may be antagonising the effects of any one or all of these opioids. Additional experiments are obviously required to determine which opioid constitutes the primary inhibitory influence on the neurons of the pPVN.

The PVN receives afferent input that contains enkephalin, dynorphins or  $\beta$ -endorphin from a variety of regions, including the PeF, ARC, VMH, Ce and NTS (see chapter 5). Of these regions, 1 session of restraint stress was found to induce a significantly increased expression of proDYN mRNA in the VMH and significantly increased levels of prepro-ENK mRNA expression in the PeF of WKY (see chapter 3). It is therefore possible that the increased expression of proDYN or prepro-ENK mRNA in the VMH or PeF leads to an increased release of dynorphins or enkephalins in the PVN of WKY during restraint. Moreover, it is the actions of these opioid peptides that naloxone may be inhibiting in the study outlined in chapter 5.

Stimulation of  $\mu$ -opioid receptors in the PVN of unstressed rats produces significant increases in HR, BP and catecholamine release; however, intra-PVN administration of naloxone does not alter HR, BP or the plasma levels of NAdr and adrenaline, suggesting that  $\mu$ -opioid receptors are not tonically stimulated (Appel *et al.*, 1986; Kiritsy-Roy *et al.*, 1986; Bachelard & Pitre, 1995). Furthermore, studies have also shown that DAGO or naloxone microinjected into the PVN of restrained rats can modify HR and stress-induced adrenaline release, demonstrating that an opioid-containing pathway(s) is active in the PVN during exposure to stress (Kiritsy-Roy *et al.*, 1986). Although the effects of intra-PVN opioids on the HPA axis has not been studied, data suggest that opioids within the CNS have the capacity to modulate HPA axis activity (see chapter 5).

As suggested earlier, the PeF may be the source of the inhibitory opioid input to the PVN, and the PeF has been shown to project to the pPVN, particularly to those pPVN neurons that project to the bulbospinal cells in the medulla oblongata and the preganglionic cells in the spinal cord (Larsen *et al.*, 1994a; Pyner & Coote, 2000). The pPVN was the subregion of the PVN where an increase in neuronal activity was induced by restraint (chapter 5), and it follows that it is the pPVN where naloxone may be exerting its antagonism of the actions of the opioids released from an afferent input to the pPVN. Lesion of the PeF has been shown to attenuate the pressor component of the conditioned fear response and intra-PeF administration of CRF has produced a tachycardia and hypertension, demonstrating that the PeF has the capacity to modulate sympathetic outflow (LeDoux *et al.*, 1988; Diamant *et al.*, 1992). LeDoux and colleagues also suggested that the afferent projection from the Ce to the PeF may be contributing to the pressor response elicited by conditioned fear (LeDoux *et al.*, 1988). However, while it is possible that the projection from the PeF to the pPVN may be the next pathway in this neural circuit that modulates sympathetic outflow during stress, the PeF also projects to the spinal cord where it may exert direct effects on the IML (Swanson & Kuypers, 1980). Nevertheless, the PeF represents a target for future studies that seek to identify the source of the opioid-containing input to the pPVN that is inhibiting neuronal activity during exposure to restraint.

In addition, there appears to be a potential correlation between the elevated expression of proDYN mRNA in the VMH of WKY rats exposed to 1 session of restraint and the increased expression of Fos-ir in the PVN of restrained naloxone-treated rats compared to the saline-treated control group that were exposed to a single restraint session. The VMH projects to the PVN, and injection of the  $\kappa$ -opioid agonist U50-488H or dynorphin A (1-13) into the PVN did not elicit any significant effects on HR, BP or peripheral haemodynamics, while an increase in plasma vasopressin was observed (Swanson & Sawchenko, 1983; Tsushima *et al.*, 1993; Bachelard & Pitre, 1995). However, intra-PVN administration of dynorphin A fragments during haemorrhage can attenuate recovery and modify HR and BP, suggesting that  $\kappa$ -opioid receptors have the capacity to modulate cardiovascular and neuroendocrine parameters at the level of the PVN (Fan & McIntosh, 1994). Similar to the PeF, blockade of CRF receptors by  $\alpha$ -helical CRF in the VMH attenuated the pressor response normally induced by intra-Ce glutamate (Wu *et al.*, 1999). The function of this pathway has not been studied during stress, so it is not clear whether the Ce-VMH connection can produce the same effects on stress-induced BP responses as the Ce-PeF pathway. Thus, there is evidence to suggest that the VMH is a part of the neural circuitry that includes the Ce and PVN, and therefore has the potential to provide an inhibitory influence on the PVN during restraint through the release of dynorphin.

## 6.4 FUTURE DIRECTIONS

### 6.4.1 RAT STUDIES

The PeF and VMH represent 2 central regions that are sensitive to restraint stress (as outlined in chapter 3) and they may be producing the opioid-mediated inhibition of neuronal activity within the pPVN as observed in chapter 5. Thus, these data demonstrate the progression from the detection of neurochemical changes in discrete nuclei during restraint (as in chapters 3 and 4), to determining whether these changes have biological significance in the neural stress response through the completion of *in vivo* functional experiments (chapter 5). There are a number of experiments that can be planned from here. From a functional point of view, plasma concentrations of hormones and catecholamines, HR, BP, respiratory rate and other physiologically important parameters can be measured in restrained rats and the effect of i.c.v. naloxone on these responses can be observed and correlated with the results of the present thesis.

When completing the preliminary experiments outlined in chapter 5 to measure the effects of i.c.v. naloxone on resting MAP, an interesting observation was made. A rat that had received 3 naloxone injections during the course of the day was placed in a restraint tube, and the indwelling catheter in the tail artery meant that BP could be monitored while the rat was in the restraint tube. As demonstrated in Figure 6.1, BP did not increase at any stage while the rat was in the restraint tube, suggesting that naloxone may be attenuating the pressor response to restraint. Although a similar experiment was not completed with a rat that received i.c.v. saline alone, work in the same laboratory using telemetry probes has demonstrated that restraint does produce a hypertension (McDougall *et al.*, 2000) (also see chapter 2). In addition, it can be clearly seen that when the rat was released at the completion of the 30 min restraint period, there is a brief pressor response similar to that seen in the study by McDougall and colleagues. This pressor response, which can be associated with increased activity upon release from the tube, does not appear to be mediated by the central opioid system.

Previous studies have demonstrated that naloxone has no effect on HR and BP in unstressed normotensive rats (Gordon, 1986; Levin *et al.*, 1986; Gordon, 1990). However, i.c.v. administration of opioid agonists such as DAGO can increase BP in normotensive rats, demonstrating that opioids, particularly those with affinity at the  $\mu$ -opioid receptor, have the capacity to modulate BP, possibly through alterations of sympathetic outflow (Kiritsy-Roy *et al.*,

**FIGURE 6.1**

Original polygraph trace demonstrating the effects of i.c.v. naloxone on the cardiovascular response exhibited by a conscious WKY rat exposed to a 30 min period of restraint stress. An intra-arterial (i.a.) catheter had been implanted in the tail artery of the rat, and blood pressure (BP) was monitored via the i.a. catheter that was attached to a pressure transducer and Grass polygraph. Note that during the restraint period, there is no increase in resting BP. However, when released from the restraint tube, there is an immediate pressor response that lasts for ~1 min before returning towards resting levels. Scale bar represents 1 min.

BP (mmHg)

150  
100  
50  
0

1min  
┌  
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IN

RESTRAINT

OUT

1989). During exposure to stress, sympathetic outflow increases, and elevated HR and BP are observed (see section 1.1 and 1.2). In normotensive rats exposed to stressors such as social stress, isolation and footshock, i.c.v. naloxone attenuates the stress-induced hypertension (Florentino *et al.*, 1987; Jiménez *et al.*, 1990). Consequently, the effects of i.c.v. naloxone on the restraint-induced pressor response in WKY as shown in Figure 6.1 support these findings. Further experiments would first confirm this effect of naloxone on BP in restrained rats was reproducible. A second set of experiments would then investigate whether the naloxone-induced changes in neuronal activity in the pPVN and SON of rats exposed to restraint alters sympathetic outflow by using Doppler probes to simultaneously measure blood flow in regional vascular beds such as the renal, mesentery and hindquarters.

If i.c.v. naloxone can attenuate the pressor response to restraint through actions at the level of the PVN, this may have some important implications. The RVLM is regarded as one of the primary influences regulating BP and sympathetic outflow (Dampney, 1994); however, i.c.v. microinjection of naloxone in restrained rats did not alter Fos expression in the RVLM and other regions that can modulate BP such as the NTS. It therefore appears that another region, such as the pPVN, may be overriding the influence of the RVLM and central baroreceptor reflex circuitry on BP control during stress. The pPVN sends efferent projections to the IML, as well as collaterals to the spinal cord and RVLM (Ranson *et al.*, 1998; Shafton *et al.*, 1998; Motawei *et al.*, 1999; Pyner & Coote, 2000), suggesting that the pPVN may regulate sympathetic outflow during exposure to stress at the level of the IML, RVLM or both.

These actions of naloxone on the cardiovascular and sympathoadrenal system during restraint also suggest that the completion of experiments similar to chapter 5 using SHR may produce some interesting results. In SHR that were exposed to an air-jet stressor, administration of naloxone attenuated the pressor response (Kapusta *et al.*, 1989). Furthermore, there is evidence demonstrating that naloxone can lower BP in SHR, where sympathetic outflow is significantly elevated when compared to WKY (Delbarre *et al.*, 1982; Levin *et al.*, 1986). Chronic administration of naloxone in young SHR was shown to suppress the development of hypertension, suggesting that alterations in the central opioid system may be contributing to increased sympathetic outflow in SHR (Quock *et al.*, 1984). Thus, there is a need for studies that measure central neuronal activation using Fos immunohistochemistry or [<sup>14</sup>C]-2-DG concentrations in unstressed SHR that have received an i.c.v. microinjection of naloxone or an antagonist with a higher selectivity at a particular opioid receptor subtype. This study could then be extended to investigate the effects of i.c.v. naloxone on central neuronal activity in SHR

exposed to restraint, in conjunction with monitoring of plasma hormones, HR, BP and blood flow to various peripheral vascular beds.

From an anatomical perspective, the neural connections of the PVN and SON that are activated during the central response to restraint in both WKY and SHR can be investigated. Specifically, experiments should focus on the opioid-containing afferents to the pPVN and SON, and look at where they originate and what opioid peptides they contain. In addition, other studies should investigate the neurons inhibited by the opioids in the pPVN and whether they project to the spinal cord. A pharmacological approach can also be undertaken, by injecting antagonists selective for the  $\mu$ - and  $\kappa$ -opioid receptors to determine which opioid peptides inhibit or excite the neurons of the pPVN and SON respectively during restraint.

A similar study to chapter 5 could be completed that focuses on the central GAL system. The GAL system has not been researched as thoroughly as the opioid system, but nevertheless, the results from chapters 3 and 4 indicate that GAL may modulate the neural stress response in a variety of discrete nuclei in both WKY and SHR. Comparisons of GAL neurochemistry in the CNS of WKY and SHR have also revealed the presence of significant alterations in specific regions (see chapters 3 and 4). With the future development of agonists and antagonists specific for each GAL receptor subtype, microinjection studies in conscious unstressed and restrained normotensive and hypertensive rats will undoubtedly provide a great deal of information regarding the functional roles of GAL within the CNS.

#### 6.4.2 HUMAN STUDIES

The therapeutic potential of these results in treating and/or managing stress is difficult to assess at this early stage. The future direction for research in the stress field represents a challenge and requires an identification of specific targets for treatment. If stress were to be clinically treated with acute or chronic drug administration, suppression of the stress response should not reduce general awareness of the surroundings and the recognition of genuine threats to the patient's health. Furthermore, reflexes to physical stressors such as hypoxia, haemorrhage and pain should also be retained. Therefore, a treatment of stress should aim to minimise the side effects of prolonged activation associated with exposure to a mental stress, such as hypertension and the increased risk of additional complications associated with hypertension. Furthermore, these drug treatments should be targeted at patients who are hyperresponsive to stress, such as those with elevated BP. Many studies have been published that demonstrate this

increased sensitivity to stress in hypertensive or patients at high risk for development of hypertension (al'Absi *et al.*, 1994; al'Absi *et al.*, 1998).

Naloxone or a derivative may be used as a therapeutic drug to minimise the stimulatory effects of stress on the sympathetic nervous system in humans. Naloxone can modulate HPA axis activity in humans, and is presently being evaluated as a diagnostic tool used to investigate dysfunction of the HPA axis in various disease states (see Nye *et al.*, 1999). Naloxone has been shown to attenuate the rise in systolic BP and plasma opioids and hormones in response to a mental stress (Fontana *et al.*, 1997), demonstrating that opioids are implicated in the physiological stress response in humans. Furthermore, the same dose of naloxone produced different effects in patients with an elevated pressor response to the mental arithmetic test, suggesting that hyperreactivity to stress may be associated with alterations in the opioid system (Fontana *et al.*, 1997). Thus, further investigation of the neural stress circuitry in humans and characterisation of the modulatory effects of opioids on the cardiovascular and neuroendocrine systems will determine whether the opioid system is an appropriate target for therapeutic treatment of the stress response in humans, particular those susceptible to elevated BP.

However, naloxone has poor pharmacokinetics in humans, with an extensive first pass metabolism after oral administration (Fishman *et al.*, 1973). Naltrexone, another non-selective opioid receptor antagonist, is also extensively metabolised by the liver, but it has been preferred to naloxone for oral treatment of disorders such as alcoholism and chronic narcotic abuse in humans due to its longer half life (Crabtree, 1984; Chick *et al.*, 2000). Consequently, further research into the opioid system and development of selective antagonists and dosage regimes may improve the management and treatment of a number of human illnesses and disorders.

## 6.5 CONCLUSION

The present thesis demonstrated that a wide variety of neurochemical differences exist between the normotensive WKY and hypertensive SHR strains in the resting state. These significant variations in gene expression or receptor density in the opioid, GAL and NPY systems were detected throughout the CNS in nuclei involved in the modulation or regulation of many autonomic and physiological functions.

Exposure to a 10 day restraint paradigm, consisting of a single 60 min restraint session per day, induced significant changes in the expression of the mRNA encoding prepro-ENK, proDYN, prepro-GAL and prepro-NPY, as well as significant alterations in opioid and GAL receptor density at various levels of the CNS of WKY. The temporal response profile observed over the 10 day restraint paradigm in multiple regions clearly showed that the neural response to restraint is dynamic, with the type of response dependent on the nucleus and neuropeptide system under investigation. SHR rats also demonstrated that the neural network governing the central response to restraint is plastic, despite the presence of an atypical stress response reported in previous studies. The changes in gene expression and receptor density induced by the 10 day restraint paradigm were significantly different in many nuclei in SHR compared to WKY, suggesting that the altered sensitivity of the GAL, opioid and NPY systems to restraint in SHR may contribute to the atypical central and physiological stress response previously observed in SHR.

Furthermore, the changes in gene expression and/or receptor density in WKY that were induced by restraint and observed throughout this thesis (chapters 3 and 4) provide a strong platform for continuing the study of the opioid, GAL and NPY neuropeptide systems in the modulation of the central and physiological stress response. In chapter 5, the function of the opioid system during exposure to restraint was investigated in conscious WKY. Following blockade of central opioid receptors by i.c.v. naloxone, restraint-induced neuronal activation was significantly altered in the PVN and SON. These findings correlate with the restraint-induced changes in gene expression observed in regions such as the PeF (prepro-ENK) and VMH (proDYN), further demonstrating that the opioid system may provide a modulatory influence on the central stress response, and possibly also contribute to the central control of sympathetic outflow at the level of the PVN or the control of the release of various hormones at the level of the PVN or SON during exposure to stress.

In conclusion, the findings of the present thesis clearly show that acute and chronic stress (restraint) can induce dynamic, significant changes in the activity of a variety of central neuropeptide systems, and these changes may contribute to the modulation of a number of central and physiological components of the stress response.

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Journal  
Jər'nel

Noun - any periodical  
or magazine,  
especially one  
published by a  
learned society.

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**APPENDIX****CONFERENCE ABSTRACTS****1. 2<sup>ND</sup> WORLD CONGRESS ON STRESS, MELBOURNE, OCT 1998****CENTRAL PREPROGALANIN mRNA EXPRESSION IN HYPERTENSIVE AND NORMOTENSIVE RAT BRAIN FOLLOWING RESTRAINT STRESS**

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Galanin, a 29 amino acid neuropeptide, is thought to participate in a number of physiological processes, including central cardiovascular regulation and the response to stress (1,2). However, the role of galanin in the stress response depends on the type of stressor, with swim stress and chronic social stress eliciting different changes in preprogalanin mRNA expression in the locus coeruleus (LC) (2,3). The present study has employed in situ hybridization histochemistry (ISHH) to investigate changes in expression of preprogalanin (ppGAL) mRNA in the forebrain (amygdala), pons (LC) and medulla (nucleus of the solitary tract (NTS)) following exposure to stress. Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive rats (SHR) were exposed to 1 hour of restraint stress between 10am and 12pm for a period of 0 (control), 1, 3, 5, or 10 days. Specific hybridization signals to ppGAL antisense oligonucleotide probes 3'-end-labeled with [<sup>33</sup>P]- $\alpha$ dATP were visualised throughout the above regions in both strains. A significant increase in ppGAL mRNA levels was seen in WKY in the central nucleus of the amygdala (CeA) ( $p < 0.05$ ; Day 0 vs Day 1(+35%). In SHR, significant increases were observed in the CeA ( $p < 0.05$ ; Day 0 vs Day 3(+117%), 5(+88%), 10(+168%)) and supraoptic area ( $p < 0.05$ ; Day 0 vs Day 10(+213%)) and a significant reduction was seen in the LC ( $p < 0.05$ ; Day 0 vs Day 1(-19%)). These results suggest that galanin may have a role in mediating the response of rats to restraint stress. In addition, the differences in ppGAL mRNA expression following chronic stress in SHR and WKY rats may represent a differential ability to cope with restraint stress.

1 Shih, C.-D., et al. (1996) *Brain Res.*, 721, 76-82.2 Holmes, P., et al. (1995) *Pharmacol. Biochem. Behav.*, 50, 655-660.3 Austin, M., et al. (1990) *Synapse*, 6, 351-357.

**2. SOCIETY FOR NEUROSCIENCE, LOS ANGELES, USA, NOV 1998**

**EFFECT OF RESTRAINT STRESS ON DELTA AND KAPPA OPIOID RECEPTOR BINDING IN NORMOTENSIVE RAT BRAIN.** B.W. Sweerts, B. Jarrott and A.J. Lawrence\*. Dept. of Pharmacology, Monash University, Clayton, Victoria, Australia, 3168.

Opioids are implicated in many physiological processes, including the stress response, analgesia, dependence and reward (1). Some of the behavioural responses associated with stress are similar to those produced by endogenous opioid peptides. In particular, changes in delta opioid receptor binding have been observed following exposure to various stressors, and kappa opioid receptors are thought to be involved in stress-induced motor suppression (2). This study has utilised receptor autoradiography to investigate changes in density of delta and kappa opioid receptors in rat brain following exposure to acute and chronic restraint stress. Wistar-Kyoto (WKY) rats were exposed to 1 hour of restraint stress between 10am and 12pm for a period of 0 (control), 1, 3, 5, or 10 days. Sections from the forebrain (amygdala), pons (locus coeruleus) or medulla (nucleus of the solitary tract (NTS)) were incubated in buffer containing radioligands for either delta receptors ( $[^3\text{H}]$ -Naltrindole) or kappa receptors ( $[^3\text{H}]$ -U69593). Significant increases in  $[^3\text{H}]$ -Naltrindole (delta) binding were observed in the hypoglossal nucleus ( $p < 0.05$ ; Day 0 vs Day 1(+43%), basomedial amygdala ( $p < 0.05$ ; Day 0 vs Day 1(+17%), 3(+12%)), ventromedial hypothalamus ( $p < 0.05$ ; Day 0 vs Day 3(+17%)) and cortex ( $p < 0.05$ ; Day 0 vs Day 3(+7%), 5(+10%)).  $[^3\text{H}]$ -U69593 (kappa) binding decreased significantly in the medial NTS ( $p < 0.05$ ; Day 0 vs Day 3(-29%)) and increased significantly in the amygdalostriatal transition area ( $p < 0.05$ ; Day 0 vs Day 5(+16%)). These results suggest that delta and kappa opioid receptors may be implicated in mediation of the stress response in normotensive WKY rats, and that particular brain regions containing delta and kappa opioid receptors may be selectively activated by acute or chronic stress.

1 Olson, G. *et al.*, (1996). *Peptides*, 17, 1421-1466.

2 Yamada, K. and Nabeshima, T., (1995). *Behav. Brain Res.*, 67, 133-145.

### 3. AUSTRALIAN NEUROSCIENCE SOC., HOBART, JAN 1999

#### ACUTE AND CHRONIC RESTRAINT STRESS AND THE CENTRAL OPIOID SYSTEM IN NORMOTENSIVE AND HYPERTENSIVE RATS

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The present study investigated the role of the central opioid system in the response to acute and chronic restraint stress. Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats were exposed daily to 1 hour of restraint stress for 0, 1, 3, 5 or 10 consecutive days. After the appropriate restraint period, rats were decapitated and the brains removed and sectioned. *In vitro* receptor autoradiography for three opioid receptor subtypes ( $\mu$  - [ $^{125}$ I]-FK-33824,  $\delta$  - [ $^3$ H]-naltrindole and  $\kappa$  - [ $^3$ H]-U69,593) was used to measure the effect of restraint on opioid receptor density in rat brain. In addition, *in situ* hybridisation histochemistry with oligonucleotide probes was utilised to measure the central expression of preproenkephalin (ppENK) and preprodynorphin (ppDYN) mRNA following restraint. In WKY and SHR, significant changes in opioid receptor density and gene expression were observed after exposure to acute and chronic restraint. Examples of robust changes include a significant increase (+58%) in  $\mu$ -receptor density in the parabrachial nucleus of WKY after chronic restraint and a consistent, significant decrease in  $\kappa$ -receptor density in the medial amygdala by -21% in WKY and by -32% in SHR after all periods of restraint. Following an initial increase in  $\delta$ -receptor density in the commissural nucleus tractus solitarius of WKY (+12%) and SHR (+54%) after acute restraint, a significant decrease (-20% WKY, -50% SHR) was maintained in both strains after chronic restraint. In addition, a significant increase in ppDYN mRNA expression was observed in the central nucleus of the amygdala of both strains (+87%, WKY/1 day; +99%, SHR/3 days) after restraint. These results demonstrate that the opioid system is an integral component of the response to restraint stress at multiple sites within the rat central nervous system.

**4. AUSTRALIAN NEUROSCIENCE SOC., MELBOURNE, JAN 2000****ACUTE AND CHRONIC RESTRAINT STRESS AND ITS EFFECTS ON GALANIN RECEPTORS IN NORMOTENSIVE AND HYPERTENSIVE RAT BRAIN****B. W. Sweerts, B. Jarrott and A. J. Lawrence**

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Galanin, a 26 amino acid neuropeptide, has been implicated in the central response to a number of different stressors (1,2). The present study has investigated the effects of acute and chronic restraint stress on galanin receptor density in the rat central nervous system (CNS) using *in vitro* receptor autoradiography. Wistar-Kyoto (WKY; normotensive) and Spontaneously Hypertensive (SHR; hypertensive) rats were exposed daily to 1 hour of restraint stress between 10am and 12pm for 0 (control), 1, 3, 5 or 10 consecutive days. Following restraint, rats were decapitated, their brains removed and subsequently processed for galanin autoradiography using [<sup>125</sup>I]-galanin. Significant changes in galanin receptor density were observed in both WKY and SHR strains in the forebrain, pons and medulla. For example, significant decreases were observed in the medial amygdala (day 1: -41%; P<0.01), central nucleus of the amygdala (day 1: -40%; day 3: -36%; day 5: -33%; all P<0.01) and ventromedial hypothalamic nucleus (VMH; day 1: -35%; day 3: -34%; all P<0.01) of WKY. In SHR, a significant increase in galanin receptor density was observed in the VMH after 1 day of restraint (+39%; P<0.05), while significant decreases were detected in the hypoglossal nucleus (day 1: -16%; P<0.01, day 5: -15%; P<0.05, day 10: -18%; P<0.01). These results support previous evidence that has reported alterations in galanin neurochemistry in rats following exposure to stressors. Furthermore, differences in galanin receptor density between WKY and SHR rats following acute and chronic restraint may contribute to the altered stress response in hypertensive rats.

1. Sweerts, B.W., Jarrott, B. and Lawrence, A.J., (1999) *Mol. Brain Res.*, **69**, 113-123.
2. Holmes, P.V., et al., (1995) *Pharmacol. Biochem. Behav.*, **50**, 655-660.

**5. JOINT ASCEPT/BPS CONFERENCE, MELBOURNE, APR 2000****THE EFFECT OF RESTRAINT STRESS ON THE EXPRESSION OF PREPRO-NPY mRNA IN NORMOTENSIVE AND HYPERTENSIVE RAT BRAIN. BW Sweerts, B Jarrott & AJ Lawrence, Dept of Pharmacology, Monash Univ, Vic 3800**

Neuropeptide Y (NPY) is reported to have a role in the modulation of numerous autonomic and physiological functions, including the response to stress (Krukoff et al 1999). This study utilised *in situ* hybridisation histochemistry (ISHH) to investigate the effect of restraint stress on the expression of preproNPY mRNA in the CNS of normotensive (Wistar-Kyoto (WKY)) and hypertensive (Spontaneously Hypertensive (SHR)) rats. Rats from both strains were exposed daily to 1 hour of restraint stress for 0 (control), 1, 3, 5 or 10 consecutive days. A standard ISHH protocol was employed to visualise specific hybridisation signals to a preproNPY antisense oligonucleotide probe. Significant differences in basal (day 0) preproNPY expression were observed only in the hypothalamic arcuate nucleus (ARC), with preproNPY mRNA levels increased by 64% in the SHR ARC ( $P < 0.05$ ) as previously observed (McLean et al 1996). Restraint stress resulted in significant changes in preproNPY mRNA expression in brain nuclei in both strains. In WKY, a significant increase (+81%;  $P < 0.05$ ) was observed in the ARC after 1 day of restraint, and this expression returned to baseline levels after continued restraint. In SHR, acute restraint (1 day) produced a significant decrease (-33%;  $P < 0.05$ ) in the expression of preproNPY mRNA in the caudal ventrolateral medulla. In addition, 5 days of restraint elicited a significant increase (+92%;  $P < 0.05$ ) in preproNPY mRNA expression in the locus coeruleus. These results support the hypothesis that NPY is implicated in the central neural response to restraint stress. Furthermore, differences in preproNPY mRNA expression observed between WKY and SHR rats following restraint may contribute to the altered stress response previously observed in hypertensive rats (McDougall et al 2000). These data, together with previous studies (Sweerts et al 1999), implicate neuropeptides as potential modulators of the behavioural and autonomic responses to psychological stress.

Krukoff TL et al (1999) *J. Neuroendocrinol*, 11, 715-23

McDougall SJ et al (2000) *Hypertension*, 35, 126-129

McLean KJ et al (1996) *Mol. Brain Res*, 35, 249-259

Sweerts BW et al (1999) *Mol. Brain Res*, 69, 113-123

**6. AUSTRALIAN NEUROSCIENCE SOC., BRISBANE, JAN 2001****OPIOIDERGIC MODULATION OF THE NEURAL RESPONSE TO ACUTE RESTRAINT****B.W. Sweerts, B. Jarrott and A.J. Lawrence**

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Restraint stress induces alterations in opioid precursor gene expression and receptor density in multiple brain regions. The present study aimed to further characterise the role of the central opioid system in the neural response to acute restraint. Male rats were anaesthetised (methohexitone; 60 mg/kg i.p.) and guide cannulae were implanted into the lateral ventricle. Following recovery, the non-selective opioid receptor antagonist naloxone (100 nmol) or saline was administered intracerebroventricularly (i.c.v.) immediately preceding exposure to a 60 min restraint stress. Rats were subsequently anaesthetised (pentobarbitone, 60 mg/kg i.p.), perfusion fixed, their brains removed, sectioned and subjected to a standard immunohistochemistry procedure to visualise the presence of Fos protein. In non-stressed rats, naloxone had no impact upon Fos expression. In contrast, acute restraint resulted in a robust expression of neuronal Fos in numerous nuclei, including the paraventricular nucleus of the hypothalamus (PVN), supraoptic nucleus (SON), periaqueductal gray, locus coeruleus and nucleus of the solitary tract. Administration (i.c.v.) of naloxone prior to restraint significantly increased the number of Fos positive cells in the PVN (+38%) and attenuated the number of Fos positive cells in the SON (-61%;  $P < 0.05$ ; student's unpaired *t*-test). Naloxone had no effect on Fos production in any other central region where cells were quantified. These results demonstrate that opioids have may modulate the neural response to restraint stress. Furthermore, as the PVN and SON are integral components of the central circuitry regulating neuroendocrine function, these results suggest that the central opioid system may be implicated in the regulation of restraint stress-induced hormone release and hypothalamo-pituitary-adrenal axis function.

**7. EXPERIMENTAL BIOLOGY, ORLANDO, FLORIDA, USA, APR 2001****CENTRAL ADMINISTRATION OF NALOXONE MODULATES RESTRAINT STRESS-INDUCED ACTIVATION OF HYPOTHALAMIC NUCLEI**

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Central administration of naloxone attenuates stress-induced hypertension (Kapusta et al., 1989). The present study aimed to further characterise the role of opioids in the neural response to acute restraint. Male Wistar-Kyoto rats were anaesthetised (methohexitone; 60 mg/kg i.p.) and guide cannulae were implanted into the lateral ventricle. Following recovery, the non-selective opioid receptor antagonist naloxone (100 nmol) or saline was administered intracerebroventricularly (i.c.v.) immediately preceding exposure to 60 min restraint stress. Rats were subsequently anaesthetised (pentobarbitone; 60 mg/kg i.p.), perfusion fixed, their brains removed, sectioned and subjected to a standard immunohistochemistry procedure to visualise the presence of Fos protein. In non-stressed rats, naloxone had no impact upon Fos expression. In contrast, acute restraint resulted in a robust expression of neuronal Fos in numerous nuclei, including the parvocellular paraventricular nucleus of the hypothalamus (PVN), supraoptic nucleus (SON), locus coeruleus (LC), rostral and caudal ventrolateral medulla (RVLM / CVLM) and nucleus tractus solitarius (NTS). Naloxone (i.c.v.) prior to restraint significantly increased the number of Fos positive cells in the parvocellular PVN (+38%) and attenuated the number of Fos positive cells in the SON (-61%;  $P < 0.05$ ; student's unpaired *t*-test). Naloxone had no effect on Fos production in CVLM, RVLM, NTS, LC or any other region examined. These results demonstrate that endogenous opioids may modulate the neural response to restraint stress, acting at the level of the hypothalamus. Furthermore, these results suggest that the central opioid system may be implicated in the regulation of restraint stress-induced hormone release and hypothalamo-pituitary-adrenal axis function and /or sympathetic outflow.

Kapusta, DR, Jones, SY, & DiBona, GF (1989) Hypertension, 13, 808-816.

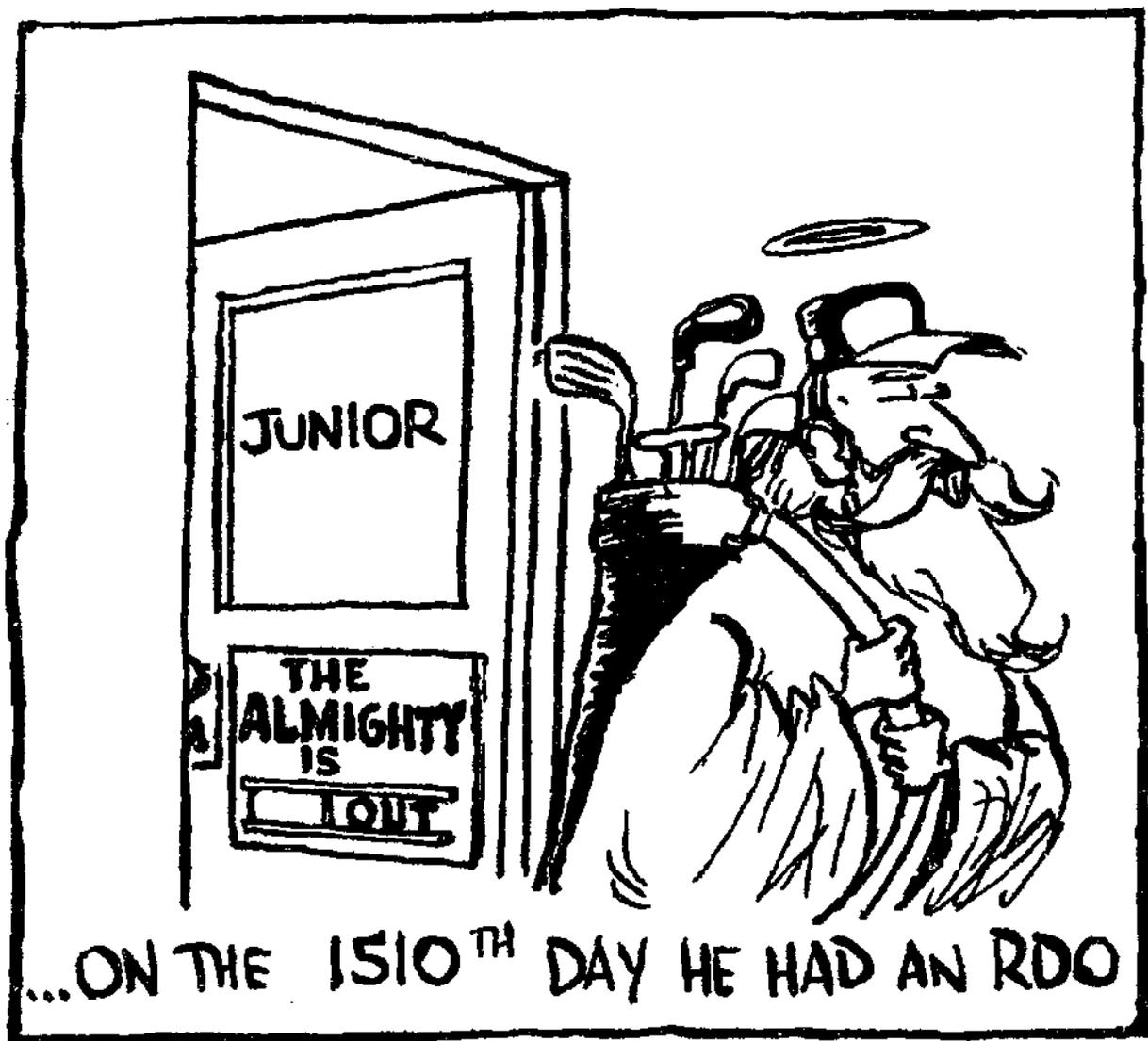
**8. 6<sup>TH</sup> INTERNATIONAL NPY CONFERENCE, SYDNEY, APR 2001****CENTRAL PREPRO-NPY mRNA EXPRESSION FOLLOWING EXPOSURE TO RESTRAINT STRESS IN THE NORMOTENSIVE AND HYPERTENSIVE RAT**

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Neuropeptide Y (NPY) has been implicated in the central regulation of a variety of autonomic and physiological functions, including the response to stress (Krukoff *et al.*, 1999). Using a standard *in situ* hybridisation histochemistry protocol, the present study quantified expression of the prepro-NPY transcript in selected brain regions of normotensive Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats that had been exposed to 1 hour of restraint stress for 0 (control), 1, 3, 5 or 10 consecutive days. Acute and chronic restraint stress produced significant changes in prepro-NPY mRNA expression in specific central regions in both strains. In WKY, acute restraint induced a significant increase in prepro-NPY mRNA levels in the arcuate nucleus (+81%;  $P < 0.05$ ) that recovered and returned to baseline levels following continued exposure to the same stressor. In the SHR strain, a significant decrease of -33% was detected in the caudal ventrolateral medulla after 1 day of restraint, while 5 days of restraint produced a significant increase in prepro-NPY mRNA expression (+92%) in the locus coeruleus ( $P < 0.05$ ). Thus, the sensitivity of the NPY-ergic system to restraint supports previous evidence implicating NPY in the central stress response. Furthermore, the difference in the response of NPY-ergic neurons to acute and chronic restraint between WKY and SHR rats may contribute to the well-documented altered stress response exhibited by hypertensive rats (McDougall *et al.*, 2000). The results of the present study also provide strong evidence implicating neuropeptides such as NPY in the modulation of the neural stress response and associated behavioural and autonomic responses to psychological stressors.

Krukoff TL *et al* (1999) *J. Neuroendocrinol*, 11, 715-23McDougall SJ *et al* (2000) *Hypertension*, 35, 126-129



*Do what you love and believe in,  
& success will come naturally.*