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MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON.....20 December 2002.....
.....[redacted].....
for Sec. Research Graduate School Committee

CORRIGENDA

Page iv line 10 "analysis of variance" should read "one way analysis of variance"
Page vi line 5. "The mRNA expression of APP protein and a number of proteins..." should read "The expression of mRNAs for APP and a number of proteins..."
Figure 1 figure legend should read "Schematic representation of the mature nicotinic acetylcholine receptor (nicR) and possible nicR subtypes constructed from different nicR protein subunits. (A) α and β subunits assemble with different stoichiometry to form a functional membrane bound receptor that channels Na^+ and Ca^{2+} from the extracellular space through a central pore to the intracellular space. (B) While muscle type nicRs have a clearly defined subunit composition (muscle nicRs can also contain ε subunits); neuronal nicRs can either be heteromeric like the α4β2 subtype or homomeric like the α7 subtype. The α4β2 and α7 nicR subtypes are the major nicRs found in mammalian brain."
Page 9 line 9. "...σ subunits found peripherally in muscle nAChR." should read "...ε subunits found peripherally in muscle nAChR."
Page 9 line 11 "...localised to sensory tissue; fig 1.0b" should read "...localised to sensory tissue (Lukas et al, 1999) "
Page 14 line 1. "some NMDAR subunits ..." should read "some NMDAR subtypes..."
Page 14 line 5. "hypothesized role...." should read "has lead to the hypothesis that these receptors....
Page 14 line 6 "pathogenisis" should read "pathogenesis"
Page 15 line 22 "Schubert et al 1989 should instead be "Schubert & Behl 1993"
Page 23 line 4 "A 10X PBS ... (pH 7.4)" should read "A 10X PBS is 1.3M NaCl, 70mM Na_2HPO_4 and 30 mM NaH_2PO_4 (pH 7.4)
Page 24 line 19 "...therefore 'pan' probes and do will not distinguish between ..." should read "...therefore 'pan' probes do not distinguish between ..."
Page 26 line 9 and Page 46 line 6. For "counter" read "Packard TriCarb scintillation spectrometer"
Page 26 line 9 "Counts..." should read "The level of radioactivity..."
Page 26 line 18. The composition of "minimalist hybridization buffer solution" is 50% formamide, 4X SSC, 10% dextran sulphate. SSC is 3M NaCl and 0.3M Na citrate (pH 7.0)
Page 26 line 18 "...vortexed..." should read "...mixed on a Vortex mixer..."
Page 30 line 2 "greyscales" should read "grey scales"
Page 43 Animal ethics considerations: All experiments were carried out according to the NHMRC Guidelines for the care and use of animals approved by the Monash University Animal Experimentation Ethics Committee
Page 45 line 14 "ph" should read "pH"
Page 46 line 6 "spectrophotometer" should read "spectrometer"
Page 90 line1 "affect" should be "effect"
Page 92. Animal ethics considerations: The animals recovered over a period of 1 hour. Animals were monitored daily over the post-operative recovery periods until they were killed
Figure 4.9 and Figure 4.13 The greatest density of α7 nAChR transcripts was found in the Ca3 region of the hippocampus while the greatest density of ^{125}I-BGTbinding was in the Dg of the hippocampus. This is not unusual as in situ hybridisation is limited to detecting subunit transcripts at the level of the cell body. Autoradiography of ^{125}I-BGTbinding can detect receptors at the level of the cell body or axons or dendrites. This is an important concept to bear in mind when comparing subunit expression (using in situ hybridisation histochemistry) with mature receptor localisation (using receptor autoradiography)
Page 162 line 20 "Relatively little, however, is known about the specific nature of interaction between Aβ peptides and cholinergic neurons" should read "Up until 2000, little was known about the specific nature of interaction between Aβ peptides and cholinergic neurons, other than early studies that suggested that Aβ peptides could interact with neuronal nicotinic receptors (Livett et al., 1993)."
Page 163 line 22 "methyllcaconitite" should read "methyllycaconitine"
Figure 6.4 and 6.5 The infusion of β-Amyloid (1-40) rate is 300 pmol/day. These results show the effects following an in vivo chronic treatment with β-Amyloid (1-40). Figure 6.3 shows the data for the direct in vitro effects of β-Amyloid (1-40)
Page 199 line 24 "α3β3α4" should read "α3β3β4"
Page 199 line 25 "compare" should read "compared"
Page 200 line 11 "raise the notion" should read "suggest that"
Page 230 line 25 "... the normal functioning of cholinergic independently of a ..." should read "...the normal functioning of cholinergic neurons independently of a ..."
Page 256 et seq The phrase "...[see comments]..." should be deleted from within the citation on pages. 256, 259, 261, 265, 284, 298, 300, 301, 302
Page 277line 2 "Behavioural" should read "Behavioral"
Page 294 line 3 "BMJ" should read "Brit. Med. J"
Page 306 line 15 "contempory" should read "contemporary "
Page 309 line 20 The phrase "...[In Process Citation]..." should be deleted from within the citation

The references below cited throughout the thesis should be amended as follows:-

"Boeckman et al 1994 should read" Boeckman & Aizenman 1994"
"Braak et al 1991" should read "Brosak and Braak 1991"
"Clark et al 1993" should read "Clark & Goate, 1993"
"Collingridge et al 1995" should read "Collingridge & Bliss 1995"
"Conroy et al 1995 should read "Conroy & Berg 1995"
"Davies et al 1976" should read "Davies & Maloney 1976"
"DeKosky et al 1990" should read "DeKosky & Terry 1994"
"Engelborghw et al 1997" should read "Engleborghts & De Deyn 1997"
"Flynn et al 1986" should read "Flynn &Mash 1986"
"Glenner et al 1984" should read "Glenner & Wong, 1984"
"Henke et al 1983" should read "Henke & Lang 1983"
"Johnson et al 1987" should read "Johnson & Ascher 1987"
"Kaiser et al 1998b should read "Kaiser & Wonnacott 1998b"
"Koelle et al 1949" should read "Koelle & Friedenwald 1949"
"Kovaks et al 1998" should read "Kovaks and Tanzi 1998"
"Loiacono et al 1990" should read "Loiacono & Mitchelson 1990"
"Loiacono et al 1999" should read "Loiacono & Gundlach 1999"
"Luetje et al 1991 should read "Luetje & Patrick 1991"
"Magnusson et al 1993" should read "Magnusson & Cotman 1993"
"Masliah et al 1994" should read "Masliah & Terry 1994"
"McDonald et al 1990" should read "Mc Donald & Johnston 1990"
"McGehee et al 1995b" should read "McGehee & Role 1995b"
"Nordberg et al 1986" should read "Nordberg & Winblad 1986"
"Nunan et al 2000" should read "Nunan & Small 2000"
"Perry et al 1995b" should read "Perry & Keller, 1995b"
"Racchi et al 1999" should read "Racchi & Govoni, 1999"
"Role et al 1996 should read "Role & Berg 1996"
"Saransaan et al 1995" should read "Saransaan & Oja 1995"
"Tanahashi et al 1998" should read "Tanahashi & Tabira 1998"
"Tariot et al 1996" should read "Tariot & Schneider 1996"
"Terry et al 1970" should read "Terry & Wisniewski 1970"
"Utas et al 1997" should read "Ulas & Cotman 1997"
"Vizi et al 1999" should read "Vizi & Lenvai 1999"
"Warpman et al 1995" should read "Warpman & Nordberg, 1995"
"Wisden et al 1994" should read "Wisden & Morris 1994"

Additional references to be included in bibliography:-

Livett, BG, Cheung, S and Small DH (1993) An amyloid peptide, bA4 25-35, mimics the function of substance P on modulation of nicotine-evoked secretion and desensitization in cultured bovine adrenal chromaffin cells. J. Neurochem. 60: 1163-1166.

Lukas, R.J., Changeux, J.P., Le Novera, N., Albuquerque, E.X., Balfour, D.J., Berg, D.Jk, Bertrand, D., Chiappinelli, V.A. Clarke, P.B., Collins, A.C., Davie, J.A., Gradiy, S.R., Kellar, K.J., Lindstrom, J.M., Marks, M.J., Quick, M., Taylor , P.W. and Wonnacott, S. (1999) International Union of Pharmacology XX, Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. Pharmacol. Rev. 51: 397-401.

Receptor and Neurochemical Changes in Models of Alzheimer-like Neuropathology

Submitted in total fulfilment of the requirements for the degree of
Doctor of Philosophy

by

LACHLAN H THOMPSON

Department of Pharmacology
Faculty of Medicine, Nursing & Health Sciences

January 2002

Declaration

This is to certify that this thesis –

i. contains no material which has been accepted for the award of any other degree or diploma,

ii. comprises only my own work and that due acknowledgement has been made in the text to any other material used,

iii. is no more than 100 000 words in length, exclusive of tables, figures, footnotes and references.

Lachlan Thompson

[redacted]

January 2002

Acknowledgements

The completion of this thesis was only made possible with the support of many people.

Firstly, to my honours and PhD supervisor, Dr. Richard Loiacono, for all his support, guidance and generosity and for giving me the opportunity to complete my tertiary education as part of his research group. Thank you. Also to Richard's wife, Jenny, thank you for all your generous hospitality along the way!

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Like most people I guess, during my time at University, in addition to my course work, I learnt a great deal about life. In this context, I would particularly like to acknowledge Lachlan Noble and Tao Browne.

I would also like to acknowledge the friendship and support of Tim and Merryn Callaghan during my PhD candidature. Merryn thanks for offering to type up my thesis (!!!).

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None of my tertiary studies would have been possible without the unconditional and overwhelming generosity, love and support of my parents, Bruce and Susan Thompson. I will always be grateful, and would like to dedicate my completed thesis to both of them. Thank you.

Finally, I would like to thank Jo. All that I have learnt in my time at University cannot compare to what I have learnt in the time I have spent with her. The work presented here would be worth nothing to me without her.

Abbreviations

Abbreviations commonly used throughout this thesis include –

α_7ODN – antisense oligodeoxyribonucleotide probe targeted to α_7 nAChR mRNA
α-BGT – α-bungarotoxin
Aβ – β-amyloid
AChE – acetylcholine esterase
AD – Alzheimer's disease
ANOVA – analysis of variance
ApoE – Apolipoprotein E
APP – amyloid precursor protein
APP-ODN – antisense oligodeoxyribonucleotide probe targeted to APP695 mRNA
BACE – β-secretase APP cleaving protein
$[Ca^{2+}]_i$ – intracellular calcium concentration
Ca1-3 – fields of the hippocampus
ChAT – choline acetyltransferase
CSF – cerebrospinal fluid
EtOH – ethanol
EPSPs – excitatory post-synaptic potentials
FAD – familial Alzheimer's disease
GABA – γ-aminobutyric acid
GAD – glutamic acid decarboxylase
HACU – high affinity choline uptake
ISHH – *in situ* hybridisation histochemistry
LTP – long term potentiation
MBN – magnocellular basal nucleus
mis-ODN – mis-sense oligodeoxyribonucleotide
nAChR(s) – nicotinic acetylcholine receptor(s)
nbM – nucleus basalis of Meynert
NGF – nerve growth factor
NFTs – neurofibrillary tangles
NMDA(R) – N-methyl-D-aspartate (receptor)
nNOS – neuronal nitric oxide synthase
NO – nitric oxide
NOS – nitric oxide synthase
ODN – oligodeoxyribonucleotide
OPCs – oligodendrocyte precursor cells
PBS – phosphate buffered saline
PET – positron emission tomography
PHFs – paired helical filaments
PKC – protein kinase C
PS1 – presenilin 1
PS2 – presenilin 2
ROD – relative optical density
sAPP(s) – secreted APP(s)
SSC – sodium citrate/sodium chloride
TACE – tumor necrosis factor-α-converting enzyme
VZ – ventricular zone

Abstract

The expression of receptors and other neurochemical markers has been examined, predominately using the techniques of receptor autoradiography and *in situ* hybridisation histochemistry, in rat models of neurological changes known to be present in Alzheimer's disease (AD).

Cholinergic neurons are thought to be particularly susceptible to the disease and therefore the quantification of cholinergic markers, particularly nicotinic receptors (nAChRs), in rat models of AD-like neuropathology has been a major focus of the work presented here.

Chronic intracerebroventricular infusions of the β-amyloid peptide caused a significant reduction in the density of markers for high affinity choline uptake sites, without affecting choline acetyltransferase or cholinesterase levels. The data suggests that β-amyloid peptides may modulate cholinergic neuron function independently of any direct neurotoxicity. This effect was potentiated by chronic nicotine treatment at doses similar to those experienced by smokers, and brings into question the hypothesis that smoking may be protective in relation to AD.

The loss of nicotinic receptors in AD is a persistent feature and one that has been correlated with the cognitive deficits associated with the disease. The expression of mature nAChRs and their corresponding subunit mRNAs was differentially affected by different models of AD-like neuropathology. While chronic β-amyloid infusions and selective lesioning of the cholinergic basal forebrain, using 192-IgG-saporin, had little effect; non-specific excitotoxic lesioning of basal forebrain nuclei resulted in a significant increase in the mRNA expression of the α_4, β_2 and α_7 subunit mRNAs.

The expression of N-methyl-D-aspartate receptor subunit mRNAs was also differentially affected by the different pathological models used here. While selective

lesioning of cholinergic basal forebrain neurons, using 192-IgG-saporin, caused an up-regulation of the NR2A subunit, excitotoxic lesioning of these neurons caused an increase in NR1 and NR2B mRNA expression. Evidence has been presented suggesting that β-amyloid peptides may directly activate NMDARs.

The mRNA expression of amyloid precursor protein (APP) and a number of proteins thought to be involved in its metabolism has been examined. Evidence has been presented suggesting there may be an important relationship between the α_7 nAChR subtype and the proteolytic processing of APP. Transient knockdown of the α_7 nAChR subunit protein resulted in a significant increase in the expression of mRNA for proteins involved in the processing of APP along the non-amyloidogenic pathway, including tumour necrosis factor-α-converting enzyme (TACE) and the presenilins (PS1 & PS2). Transient knockdown of APP resulted in a significant reduction in the density of the α_7 nAChR subtype.

The data presented here demonstrates that different neuropathological changes that occur in AD, such as increased β-amyloid levels and degeneration of the cholinergic basal forebrain, differentially affects the expression of a variety of neurochemical markers. The relative importance of each neuropathological model used here, and the resulting neurochemical changes, have been discussed with regard to both the further progression and potential therapy of the disease.

Table of Contents

DECLARATION	I
ACKNOWLEDGEMENTS	II
ABBREVIATIONS	IV
ABSTRACT	V
TABLE OF CONTENTS	VII
CHAPTER 1: GENERAL INTRODUCTION	1
CHAPTER 2: GENERAL METHODOLOGY	22
2.1 IN SITU HYBRIDISATION HISTOCHEMISTRY	23
2.2 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY	28
2.3 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY	28
2.4 ACHE HISTOCHEMISTRY	29
2.5 QUANTITATION	29
CHAPTER 3: THE EFFECT OF IN VIVO β-AMYLOID & CHRONIC NICOTINE TREATMENT ON MULTIPLE NEUROCHEMICAL MARKERS IN THE RAT BRAIN	33
3.1 INTRODUCTION	34
3.2 METHODS	43
3.2.1 TREATMENT OF ANIMALS AND TISSUE PREPARATION	43
3.2.2 ACHE HISTOCHEMISTRY	45
3.2.3 RADIOENZYMATIC CHAT QUANTIFICATION	45
3.2.4 [^{3}H]-HEMICHOLINIUM AUTORADIOGRAPHY	46
3.2.5 IN SITU HYBRIDISATION HISTOCHEMISTRY	47
3.2.6 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY	47
3.2.7 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY	47
3.28 QUANTITATION	48
3.3 RESULTS	49
3.3.1 AChE LEVELS	49
3.3.2 ChAT ACTIVITY	50
3.3.3 [^{3}H]HEMICHOLINIUM AUTORADIOGRAPHY	51

3.3.4 nAChR Subunit mRNA Expression 54
3.3.5 [^{3}H]Epibatidine Autoradiography 56
3.3.6 [^{125}I]α-Bungarotoxin Autoradiography 58
3.3.7 NMDA Receptor Subunit mRNA Expression 60
3.3.8 APP695, BACE, PS1, PS2 and TACE mRNA Expression 65

3.4 Discussion 70

Chapter 4: The effect of selective degeneration of the cholinergic basal forebrain system on multiple neurochemical markers in the rat brain 81

4.1 Introduction 82

4.2 Methods 92

4.2.1 Treatment of Animals and Tissue Preparation 92
4.2.2 AChE Histochemistry 93
4.2.3 In Situ Hybridisation Histochemistry 93
4.2.4 [^{3}H]Epibatidine Autoradiography 93
4.2.5 [^{125}I]α-Bungarotoxin Autoradiography 93
4.2.6 Quantitation 94

4.3 Results 95

4.3.1 AChE Histochemistry 95
4.3.2 nAChR Subunit mRNA Expression 98
4.3.3 [^{3}H]Epibatidine Autoradiography 101
4.3.4 [^{125}I]α-Bungarotoxin Autoradiography 102
4.3.5 NMDA Receptor Subunit mRNA Expression 104
4.3.6 nNOS mRNA Expression 107
4.3.7 APP695, BACE, PS1, PS2 and TACE mRNA Expression 109

4.4 Discussion 115

Chapter 5: The effect of excitotoxic lesioning of the basal forebrain on multiple neurochemical markers in the rat brain 126

5.1 Introduction 127

5.2 Methods 134

5.2.1 Treatment of Animals and Tissue Preparation 134
5.2.2 AChE Histochemistry 135
5.2.3 In Situ Hybridisation Histochemistry 135
5.2.4 [^{3}H]Epibatidine Autoradiography 135
5.2.5 [^{125}I]α-Bungarotoxin Autoradiography 135
5.26 Quantitation 136

5.3 RESULTS 137

5.3.1 AChE HISTOCHEMISTRY 137
5.3.2 nAChR SUBUNIT mRNA EXPRESSION 138
5.3.3 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY 140
5.3.4 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY 142
5.3.5 NMDA RECEPTOR SUBUNIT mRNA EXPRESSION 144
5.3.6 APP695, BACE, PS1, PS2 AND TACE mRNA EXPRESSION 146

5.4 DISCUSSION 149

CHAPTER 6: INVESTIGATIONS INTO POSSIBLE INTERACTIONS BETWEEN β-AMYLOID AND THE α7 nAChR IN VITRO AND IN VIVO 161

6.1 INTRODUCTION 162

6.2 METHODS 164

6.2.1 TREATMENT OF ANIMALS AND TISSUE PREPARATION 164
6.2.2 [^{125}I]α-BUNGAROTOXIN BINDING TO MEMBRANES 165
6.2.3 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY 166
6.2.4 IN SITU HYBRIDISATION HISTOCHEMISTRY 167

6.3 RESULTS 168

6.3.1 [^{125}I]α-BUNGAROTOXIN BINDING TO MEMBRANES 168
6.3.2 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY 169
6.3.3 [^{125}I]α-BGT BINDING AND α_7 SUBUNIT EXPRESSION FOLLOWING β-AMYLOID$_{(1\text{-}40)}$ INFUSIONS 171

6.4 DISCUSSION 173

CHAPTER 7: TRANSIENT KNOCKDOWN OF THE α7 nAChR SUBUNIT PROTEIN IN THE RAT BRAIN AFFECTS THE RXPRESSION OF OTHER nAChR SUBUNITS AS WELL AS CERTAIN PROTEINS INVOLVED IN THE PROTEOLYTIC PROCESSING OF APP 177

7.1 INTRODUCTION 178

7.2 METHODS 184

7.2.1 TREATMENT OF ANIMALS AND TISSUE PREPARATION 184
7.2.2 IN SITU HYBRIDISATION HISTOCHEMISTRY 185
7.2.3 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY 185
7.2.4 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY 186

7.2.5 QUANTITATION 186

7.3 RESULTS 187

7.3.1 α_7 nAChR SUBUNIT mRNA EXPRESSION AND RECEPTOR LEVELS 187

7.3.2 NON-α_7 NACHR SUBUNIT MRNA EXPRESSION 190
7.3.3 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY 193
7.3.4 mRNA EXPRESSION OF APP695, BACE, PS1, PS2 AND TACE 195

7.4 DISCUSSION 198

CHAPTER 8: TRANSIENT KNOCKDOWN OF THE APP695 PROTEIN IN THE RAT BRAIN AFFECTS α_7 NACHR DENSITY AS WELL AS THE EXPRESSION OF TACE mRNA IN CERTAIN TELENCEPHIC AREAS 207

8.1 INTRODUCTION 208

8.2 METHODS 211

8.2.1 TREATMENT OF ANIMALS AND TISSUE PREPARATION 211
8.2.2 IN SITU HYBRIDISATION HISTOCHEMISTRY 212
8.2.3 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY 212
8.2.4 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY 213
8.2.5 QUANTITATION 213

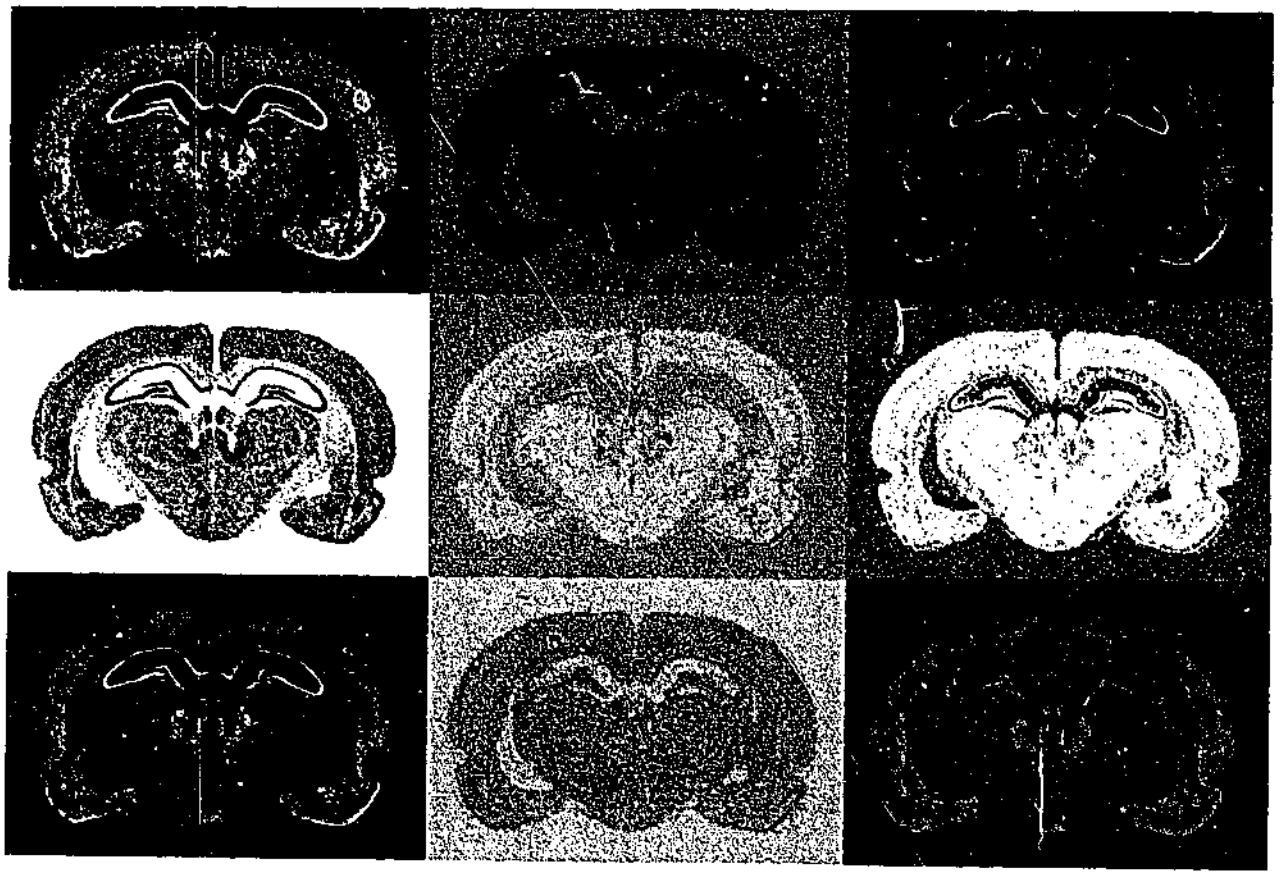
8.3 RESULTS 214

8.3.1 mRNA EXPRESSION OF APP695 214
8.3.2 nAChR SUBUNIT mRNA EXPRESSION 215
8.3.3 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY 217
8.3.4 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY 219
8.3.5 mRNA EXPRESSION OF BACE, PS1, PS2 AND TACE 221

8.4 DISCUSSION 223

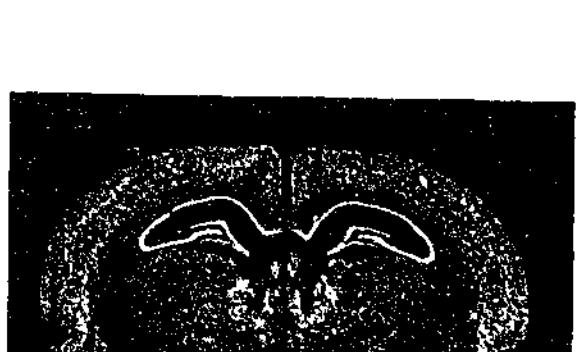
CHAPTER 9: GENERAL DISCUSSION 229

REFERENCES 243



Chapter 1

General Introduction



"*In Alzheimer's [disease] the mind dies first: Names, dates, places-the interior scrapbook of an entire life-fade into mists of nonrecognition.*" - Matt Clark, 1984.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting approximately 10% of the population over 65 years of age and up to 50% over the age of 85 (Hof et al., 1995). Clinically, AD is initially characterised by cognitive decline and the impairment of the ability to form new memories. In 1907, describing the behaviour of a 54 year old woman, Dr. Alois Alzheimer wrote:

"*When the doctor showed her some objects, she first gave the right name for each, but immediately afterwards she had already forgotten everything ... In her conversation, she often used confused phrases, single paraphrasic expressions (milk-jug, instead of cup). Sometimes she would stop talking completely. She evidently did not understand many questions (and) ... she did not remember the use of particular objects.*" (Alzheimer, 1907)

Dr. Alzheimer would later perform an autopsy on the patient's brain and discover the neuritic plaques and neurofibrillary tangles (NFTs) that are now regarded as pathological hallmarks of AD. The presence of these neuritic plaques and NFTs represent the neuropathological basis on which AD brains are distinguished from normal aged brains at autopsy.

Aggregates of 7-10 nm filaments form in the extracellular space within the brains of subjects with AD. These filaments are comprised of an insoluble form of a 4 kDa peptide termed β-amyloid (Aβ) (Glenner et al., 1984; Masters et al., 1985) which is derived through both constitutive and stimulated proteolytic processing of the larger amyloid precursor protein (APP) (Haass et al., 1993b; Selkoe, 1994a; Selkoe et al., 1988). In AD, Aβ deposition occurs diffusely throughout the parenchyma as amorphous, non-filamentous aggregates termed 'diffuse plaques' (Glenner et al., 1984) and also in the form of spherical structures with a dense inner core known as 'neuritic plaques'. Neuritic plaques are surrounded by reactive microglial cells and astrocytes and are spatially localised with

dystrophic neurites (Dickson, 1997; Terry et al., 1994). In addition to amyloid deposition, profound changes in cytoskeletal structure related to the abnormal phosphorylation of tau proteins occur in neurons and their processes in AD. These changes are associated with abnormal, highly insoluble filamentous structures called 'paired helical filaments' (PHFs) that contribute towards the formation of tangle-like inclusions in cell bodies (Terry et al., 1970). These neurofibrillary tangles (NFTs) remain in the extracellular space following the complete degeneration of the affected neuron. The relationship between amyloid plaque and NFT formation in AD brains is not well understood.

While NFTs and neuritic plaques are invariably associated with AD, the significance of these morphological changes in relation to the neurodegenerative cascade underlying the disease remains largely unknown. The neurodegenerative process is characterised by progressive neuronal and synaptic loss (Bartus et al., 1982; Davies et al., 1976; DeKosky et al., 1990; Lassman et al., 1992; Masliah et al., 1994; Storga et al., 1996; Terry et al., 1991; Terry et al., 1981) and a concomitant decrease in specific neurotransmitters including acetylcholine, γ-amino-butyric acid (GABA), glutamate, noradrenaline and serotonin (Cowburn et al., 1990; Meltzer et al., 1998; Perry et al., 1977; Storga et al., 1996; Yew et al., 1999; for review see Engelborghs et al., 1997) and their corresponding receptors (Blin et al., 1993; Cross et al., 1984; Flynn et al., 1986; Ikonomovic et al., 1997; Jansen et al., 1990; Meana et al., 1992; Pizzolato et al., 1996; Sugaya et al., 1990; Ulas et al., 1997; Warpman et al., 1995). Neuronal loss and in particular the decline in synaptic density seen in AD is strongly correlated to the development and progression of memory impairment and cognitive dysfunction (Arriagada et al., 1992a; DeKosky et al., 1990; Samuel et al., 1994; Terry et al., 1991). The relationship between the degenerative process and the clinical symptoms seen in AD is likely to be based on the selective vulnerability of certain brain regions and neuronal types to the disease process. For example, association neocortical areas are particularly

susceptible, whereas primary motor and sensory regions are less affected. The amygdala, hippocampus and parahippocampus also demonstrate a high degree of susceptibility to neuronal degeneration. In contrast, regions of the brain such as the striatum and cerebellum may show some degree of plaque formation but do not develop significant neuropathology. Due to the selective vulnerability of the cortex and hippocampus to the neurodegenerative process associated with AD, the experimental work presented in this thesis has focussed, in particular, on the analayis of these structures. There also appears to be a temporal staging of neuronal degeneration that parallels the development of the clinical symptoms seen in AD (Table 1.0; Braak et al., 1991; Zilles et al., 1995). A number of studies have shown that the neurodegenerative process in AD initiates in the entorhinal cortex layer 2 and then spreads to the hippocampus, temporal cortex, fronto-parietal region, and subcortical nuclei (Braak et al., 1991; Gomez-Isla et al., 1996; Masliah et al., 1994). Neurodegeneration of the intra-hippocampal circuitry results in disconnection between the dentate granular cells and the Ca3 region, the Ca1 region and the subiculum (Samuel et al., 1994). All of these major limbic system circuitries are critically important in learning and memory (Hyman et al., 1986). The profound loss of subcortical basal forebrain nuclei and resulting loss of cholinergic input to the neocortex seen in AD (Davies et al., 1976) has been consistently correlated with learning and memory impairments (Bartus, 1986; Bartus et al., 1982; Collerton, 1986; Fibiger, 1991; Flicker et al., 1985) and is widely regarded as one of the most significant neurological events in relation to AD symptomatology. Indeed, the cholinergic hypofunction associated with AD is the basis for the development and use of acetylcholinersterase inhibitors as the primary form of therapy for the disease. To date, three different cholinesterase inhibitors have been approved by the FDA in the USA, including tacrine, donepezil and galantamine, and the approval of others is expected to be given in the near future (Davis, 1998; Tariot et al., 1996). However, despite the enormous investment in this area of drug development there has been only limited and variable

clinical success. The effectiveness of these drugs has been limited by factors such as relatively poor brain delivery and toxicity due to non-specific targeting of cholinesterases (Greig et al., 1995). The development of new treatment strategies for AD is complicated by a lack of understanding of the mechanisms underlying the neurodegenerative process.

Clinical Symptoms	Structural Alterations
Memory impairment	Entorhinal region
Memory impairment + Emotional Disturbance	Entorhinal region + Limbic system
Memory impairment + Emotional Disturbance + Praxic and Gnostic dysfunctions	Entorhinal region + Limbic system + Association cortex
Memory impairment + Emotional Disturbance + Praxic and Gnostic dysfunctions + Motor Deficit	Entorhinal region + Limbic system + Association cortex + Motor Cortex

Table 1.0. Parallel sequence of neuropathological changes and clinical symptoms that may occur in the progression of Alzheimer's disease. Table adapted from (Zilles et al., 1995).

A number of risk factors have been associated with the development of AD, including age, genetic mutations, unknown environmental factors and apolipoprotein E (ApoE) polymorphism. The greatest risk is certainly age. The incidence of AD increases dramatically with age and extrapolations from current data suggest almost uniform susceptibility to the disease if an individual lives to the age of 120 years (Hof et al., 1995). The majority of AD cases are late in onset, lack an obvious genetic etiology and are characterised as sporadic, whereas a small percentage of cases are early in onset (<65 years old) and segregate strongly within families (FAD), suggesting a genetic etiology. Although FAD accounts for only a small percentage of all cases of AD, the finding that specific genetic mutations are a significant risk factor associated with FAD has generated a great

deal of interest because of the insight that these mutations might provide in relation to the pathology of the more common sporadic form of the disease. The mutations are found in at least three different genes on three different chromosomes: the APP gene on chromosome 21 (Goate et al., 1991), the PS-1 gene on chromosome 14 (Sherrington et al., 1995) and the PS-2 gene on chromosome 1 (Levy-Lahad et al., 1995). The significance of these mutations is discussed in greater detail in *Chapter 3, 3.1 Introduction*. In 1991, linkage to chromosome 19 was reported in families with late onset AD (Namba et al., 1991; Pericak-Vance et al., 1991). One of the genes located on this chromosome codes for the production of apolipoproteinE (ApoE). ApoE polymorphism consists of three types – ApoE ε2, ApoE ε3 and ApoE ε4, which result in six different ApoE phenotypes in the population. A number of studies have shown that the inheritance of one or two alleles of ApoE ε4 is a significant risk factor associated with the incidence of both familial and sporadic forms of AD (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993; for review see Roses, 1994).

One of the greatest challenges presented to the AD researcher is to relate the wide variety of neurochemical changes seen in AD to the aforementioned risk factors and, in particular, to establish some sort of hierarchy in terms of 'cause and effect' between specific neurochemical changes. There is certainly some degree of contention and ambiguity in the literature in regard to which changes constitute the pathological alterations that contribute to the neurodegenerative cascade and which changes are largely consequential of these pathological alterations. In this context, the work presented here has examined how various neurochemical markers are affected in rat models of specific neurodegenerative and morphological changes thought to underlie the neurodegenerative process in AD. The experimental manipulations employed included:

- Chronic intracerebroventricular Aβ infusion (Chapter 3)

- Immunotoxic lesioning of the cholinergic basal forebrain system (Chapter 4)
- Excitotoxic lesioning of the nucleus basalis of Meynert (Chapter 5)
- *In vitro* treatment with Aβ peptides (Chapter 6)
- Transient knockdown of the α_7 nicotinic receptor subunit protein (Chapter 7)
- Transient knockdown of the amyloid precursor protein (Chapter 8)

A detailed account of these models and their relevance to the pathology of AD is given in the relevant chapters. The neurochemical markers examined in relation to these models can be broadly defined as (i) cholinergic markers, (ii) N-methyl-D-aspartate receptor subunits and (iii) amyloid precursor protein (APP) and related proteins involved in the proteolytic processing of APP. The significance of these markers in relation to AD are discussed below.

CHOLINERGIC MARKERS IN AD

The cholinergic markers that have been examined in this study include choline acetyltransferase (ChAT), acetylcholinesterase (AChE) and nicotinic acetylcholine receptors (nAChRs).

In the mid-1970s three laboratories nearly simultaneously reported that the brains of AD patients exhibited a significant loss in activity of the key acetylcholine-synthesising enzyme, ChAT (Bowen et al., 1976; Davies et al., 1976; Perry et al., 1977). This finding was initially reported by Bowen *et al* (1976) who also found that the loss of ChAT activity was correlated with the cognitive impairment seen in AD patients. Reductions in AChE levels have also been consistently reported in AD (Appleyard et al., 1992; Bierer et al., 1995; Davies, 1979; Henke et al., 1983) and correlated with learning and memory

impairments (Davis et al., 1999). Both ChAT and AChE are widely used as markers for cholinergic neuron viability in animal models of neurodegeneration.

In addition to the loss of cholinergic neuronal activity, significant changes in both muscarinic and nicotinic acetylcholine receptor levels have been widely reported in AD brains. Radioligand binding experiments have provided inconsistent results in relation to how muscarinic receptor levels are affected in AD with different studies reporting either increased (Vogt et al., 1992), decreased (Claus et al., 1997; Holman et al., 1985; Ladner et al., 1999) or no change (DeKosky et al., 1992; Schroder et al., 1991) relative to age-matched controls. The development of muscarinic agonists for the treatment of AD has been largely unsuccessful. Many of these drugs have been evaluated clinically, but none have shown reliable clinical efficacy and most have been associated with a prohibitive level of side effects (Cassidy et al., 1994; Patel, 1995b). Nicotinic receptor pharmacology is an area that has proved to be far more promising. Over the past decade, epidemiological and neurochemical data has accumulated to support a potentially important role for neuronal nAChRs in AD. The evidence regarding how nAChRs are affected in AD has been more consistent compared with data on muscarinic receptors and clinical trials with nicotinic receptor agonists have so far proved more successful than earlier trials with muscarinic drugs. A variety of radioligand binding and positron emission tomography (PET) imaging studies have invariably shown a significant loss the density of nAChRs in the brains of AD patients relative to age-matched controls (Flynn et al., 1986; Guan et al., 2000; Nordberg et al., 1990; Nordberg et al., 1986; Sugaya et al., 1990; Warpman et al., 1995). Along with these results, the finding that smoking is associated with a delayed onset of AD initially reported by van Duijn and Hofman (1991) has stimulated a great deal of research into the potential use of nAChR ligands in the therapy of AD over the past decade. During that time a number of reports have described the ability of nicotine to improve certain memory-related and cognitive functions in AD patients (Jones et al., 1992;

Nordberg et al., 1998; Sahakian et al., 1989). Unfortunately nicotinic therapy is presently limited by the large number of side effects associated with non-specific stimulation of multiple nAChR subtypes and a lack of selective ligands for these subtypes.

Nicotinic receptors are excitatory ion channels constructed from a number of protein subunits assembled pentamerically around a central pore which channels Na^+ and Ca^{2+} ions (fig 1.0a; for reviews see Brioni et al., 1997; Colquhoun et al., 1997; Sargent, 1993).

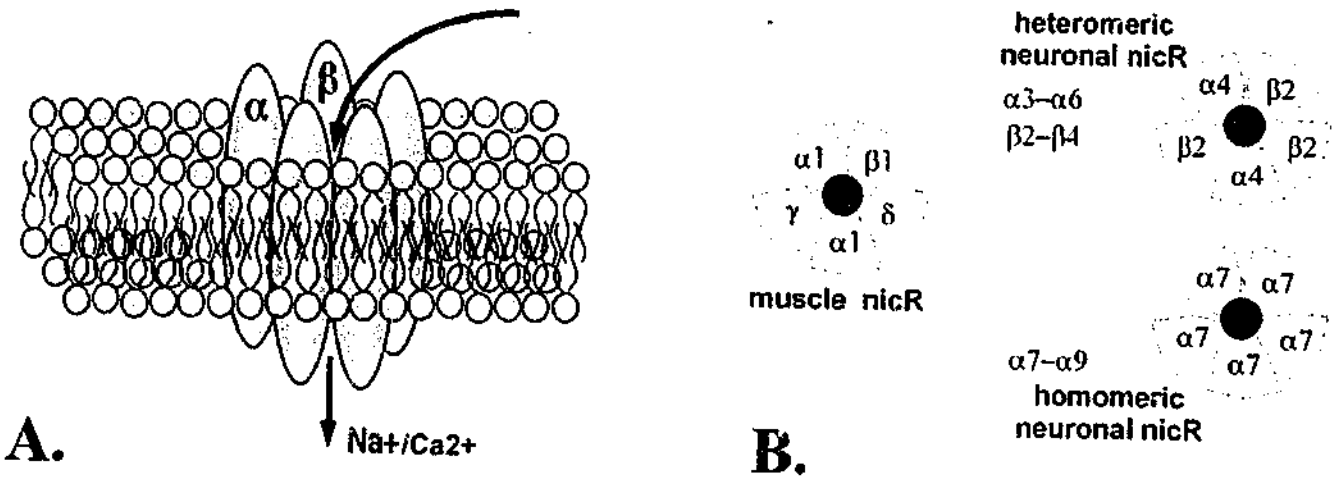


Figure 1.0. Schematic representation of the mature nAChR (A) and possible nAChR subtypes constructed from different nAChR protein subunits (B). Abbreviations: nicR, nicotinic receptor.

To date, at least 6 alpha ($\alpha_2 - \alpha_7$) and 3 beta ($\beta_2 - \beta_4$) nicotinic subunit proteins have been identified in human and rat brain. (A number of other subunits have been identified which have not been examined in this study including: the α_1, $\beta 1$, γ and σ subunits found peripherally in muscle nAChRs; the α_8 subunit found in the chick; and the α_9, and recently the α_{10} subunits localized to sensory tissue; fig. 1.0b). Corresponding rat and human nAChR subunits have a high (>70%) degree of sequence homology (McGehee et al., 1995b; Sargent, 1993). *Xenopus* oocyte transfection studies have been particularly useful in demonstrating that different combinations of nAChR subunits yield pharmacologically distinct nAChR subtypes. The subunit composition of the receptor determines its characteristics in terms of single channel properties (Papke et al., 1989), function (Patrick

et al., 1993) and response to different ligands (Luetje et al., 1991). Some of the functional receptor subtypes identified to date include $\alpha_2\beta_2$, $\alpha_3\beta_2$, $\alpha_4\beta_2$, $\alpha_3\beta_4$, $\alpha_3\beta_4\alpha_5$, $\alpha_3\beta_2\alpha_5$, $\alpha_3\beta_4\alpha_5$, α_7, α_8, and α_9 (Conroy et al., 1995; Conroy et al., 1992; Luetje et al., 1991; Role, 1992; Role et al., 1996; Vernallis et al., 1993; Wang et al., 1996; for review see Brioni et al., 1997). Physiologically, nAChRs are distributed predominately at presynaptic sites and to a lesser extent at postsynaptic and preterminal or somatic sites (Wonnacott, 1997). Evidence to date suggests that the major role for nAChRs in the brain is in the presynaptic positive modulation of neurotransmitter release (Gray et al., 1996; McGehee et al., 1995a; Wonnacott, 1997; for reviews see Kaiser et al., 1998b; Vizi et al., 1999). A number of studies have demonstrated that nAChR stimulation can enhance the release of noradrenaline, dopamine, acetylcholine, γ-aminobutyric acid (GABA) and glutamate in a nAChR subtype specific manner (Table 1.1).

One of the attractions associated with research into nAChR based therapy of AD lies in the complexity of the neuronal nAChR system and the potential to target specific neuronal systems in selected brain regions using nAChR subtype selective ligands. The major nAChR subtypes found in both rat and human brains are the heteromeric $\alpha_4\beta_2$ subtype and the homomeric α_7 subtype. The snake toxin α-bungarotoxin (α-BGT) is a selective ligand for the α_7 nAChRs and has been used widely in radioligand and autoradiographic studies to study α_7 nAChR levels and distribution. The chloroalkoloid, epibatidine, first extracted from the skin of the Ecuadorian frog *Epipedobates tricolor* (Daly et al., 1978), is commonly used to examine the density and distribution of the $\alpha_4\beta_2$ nAChR subtype. Epibatidine binding predominately reflects the presence of the $\alpha_4\beta_2$ nAChR subtype, which is the most common subtype in the brain and accounts for over 90% of α-BGT insensitive binding. While significant reductions in the density of $\alpha_4\beta_2$ have been consistently reported (Nordberg et al., 1986; Warpman et al., 1995; Whitehouse et al., 1986), studies of post-mortem AD brain tissue have found either a reduced number

(Burghaus et al., 2000; Guan et al., 2000; Wevers et al., 1999) or no significant change (Martin-Ruiz et al., 1999; Sugaya et al., 1990) in the level of α_7 nAChR subunit protein and an increase in α_7 subunit mRNA expression in AD brains relative to aged matched controls (Hellstrom-Lindahl et al., 1999).

Neurotransmitter	Region	Suggested subtype	Author
Noradrenaline	Hippocampus	$\alpha_3\beta_2$	(Sershen et al., 1997; Vizi et al., 1995)
	Hippocampus	$\alpha_3\beta_4$	(Clarke et al., 1996; Luo et al., 1998)
Dopamine	Striatum		(Grady et al., 1992; Rapier et al., 1990)
	Striatum	$\alpha_4(\alpha_5)\beta_2$	(Clarke et al., 1996)
	Striatum	$\alpha_3\beta_2$	(Kulak et al., 1997)
Acetylcholine	Hippocampus	$\alpha_4\beta_2$	(Loiacono et al., 1990; Tani et al., 1998a)
γ-aminobutyric acid	Hippocampus	$\alpha_4\beta_2$	(Alkondon et al., 1997b; Lu et al., 1998a)
Glutamate	Ventral tegmental area	α_7	(Schilstrom et al., 2000)
Serotonin	Hippocampus		(Kenny et al., 2000)

Table 1.1. A number of neuronal nAChR subtypes have been suggested to have a role in the positive modulation of the release of a variety of neurotransmitters in the brain.

Despite the extensive amount of research that has been conducted in the area of nicotinic pharmacology in regard to AD, the relationship between the nAChR loss and other neurodegenerative and morphological changes seen in AD brains is not particularly well understood. One of the aims of the work presented here has been to explore this relationship by examining how $\alpha_4\beta_2$ and α_7 nAChRs are affected at both the receptor and subunit mRNA level in rat models of specific pathological changes seen in human AD. Additionally, identifying particular nAChR subunits or subtypes as being specifically involved in certain aspects of AD pathology may play an important role in the

development of new nAChR drug therapies. At present, nAChR therapy is associated with a significant level of side effects that are probably related to the non-specific targeting of a wide range of nAChR subtypes. Clearly it would be beneficial to target only those nAChR subtype/s that mediate the therapeutic properties of nicotine in relation to AD. The neuroprotective and memory-related actions reported for novel α_7 selective nAChR ligands have indicated that this receptor subtype is a potentially important target in regard to nAChR based therapy of AD (Meyer et al., 1998b). However, relatively little is known about precisely how the α_7 nAChR mediates therapeutic and neuroprotective effects, or how a loss in the density of this receptor may be related to changes in other neurochemical markers as part of the neurodegenerative process associated with AD. The effect of transient knockdown of the α_7 nAChR subunit on the expression of other neurochemical markers that may be important in AD is another area that has been investigated as part of the work presented here.

N-METHYL-D-ASPARTATE RECEPTOR SUBUNIT EXPRESSION

N-methyl-D-aspartate receptor (NMDAR) pharmacology has been circumstantially linked to AD based on the findings that NMDARs play a crucial role in learning and memory processes and that excessive stimulation of NMDARs is associated with neurotoxicity. Further investigations have indeed shown that glutamatergic neurotransmission (Cowburn et al., 1990) and NMDAR levels (Krystal et al., 1999; Sze et al., 2001) are affected in AD brains.

NMDARs are ligand gated ionotropic receptors belonging to the same family of transmembrane ion channels as the nAChRs. The NMDARs mediate fast, excitatory glutamatergic transmission and are characterized by a high permeability to Ca^{2+} ions (MacDermott et al., 1986), a voltage dependent Mg^{2+} block (Nowak et al., 1984) and slow

gating kinetics (Johnson et al., 1987). Like nAChRs, NMDARs are constructed from a number of distinct protein subunits. In 1991, the first NMDAR subunit cDNA, NR1, was isolated by expression cloning using *Xenopus* oocytes (Moriyoshi et al., 1991). Low stringency cDNA library screening and polymerase chain reaction techniques have since been used to clone the genes for four additional subunits termed NR2A, NR2B, NR2C and NR2D (Ishii et al., 1993; Meguro et al., 1992; Monyer et al., 1992). In mammalian cells, NMDAR function is dependent on the presence of the NR1 subunit in combination with at least one other type of NR2A-D subunit (Boeckman et al., 1994; McIlhinney et al., 1996). Different subunit combinations result in pharmacologically unique NMDAR subtypes which differ in their properties in terms of: strength of the Mg^{2+} block (Monyer et al., 1994; Monyer et al., 1992); sensitivity to modulation by glycine (Ishii et al., 1993; Wafford et al., 1993); desensitization and offset decay (Kohr et al., 1994; Monyer et al., 1994; Monyer et al., 1992); and affinities for specific agonists and antagonists (Ishii et al., 1993; Monyer et al., 1994; Monyer et al., 1992; Moriyoshi et al., 1991; Wafford et al., 1993). Radioligand binding studies have led to the identification of at least four pharmacologically distinct NMDA receptor subtypes (Lynch et al., 1994). A study comparing the expression of the NR2A-D subunits and the expression of heteromeric NMDA receptor combinations in *Xenopus* oocytes concluded that these four previously identified native NMDARs differ in their NR2 composition and that the NR2 subunits significantly contribute to the anatomical and pharmacological diversity of NMDAR subtypes (Buller et al., 1994).

One of the key roles for NMDARs in the brain is in the initiation and maintenance of the activity-dependent synaptic plasticity referred to as long term potentiation (LTP) (Collingridge, 1987; Collingridge et al., 1995; Morris et al., 1986). LTP is thought to represent the physiological basis for learning and memory and is mediated by calcium flow through NMDAR channels, which ultimately leads to alterations in the strength of

interneuronal synaptic connectivity. Some NMDAR subunits appear to have a greater role in learning and memory (Tang et al., 1999). The high permeability of NMDARs to calcium is also thought to be the basis for excitotoxicity resulting from excessive NMDAR stimulation (Choi, 1988; McDonald et al., 1990). The potential for NMDARs to cause excitotoxic neuronal damage has lead to the hypothesized role of these receptors in the pathogenisis of neuropsychiatric disorders such as AD (Olney, 1989; Thomas, 1995).

At least four different laboratories studying three different non-human species (mice, rats, monkeys) have reported that the NMDAR transmitter system becomes markedly hypofunctional with advancing age (Gonzales et al., 1991; Magnusson et al., 1993; Saransaari et al., 1995; Wenk et al., 1991). There are some inconsistencies in the available literature concerning how NMDARs are affected in AD. Radioligand binding studies have shown either a significant loss in cortical and hippocampal NMDAR levels relative to age-matched controls (Greenamyre et al., 1987; Greenamyre et al., 1985) while others have reported no change (Geddes et al., 1986; Monaghan et al., 1987). At the subunit level, a recent study has reported that NR1, NR2A, and NR2B subunit proteins are significantly reduced in AD brains and that this loss in subunit proteins is correlated with cognitive deficits (Sze et al., 2001). It is not clear, however, whether this change in subunit level is reflective of a reduction in the expression of mRNA for these proteins or a result of neuronal loss.

As with many of the neurochemical changes seen in AD, the relationship between the reported changes in NMDAR subunit levels and the neurodegenerative and morphological changes seen in AD is not understood. The work presented here has attempted to identify which NMDAR subunits might be particularly important in AD and which specific pathological changes might affect these NMDAR subunits by examining how NMDAR subunit mRNA expression is affected in the aforementioned rat models of AD-like neurodegeneration. The variability in the distribution and pharmacological

characteristics of the different NMDAR subtypes provides a complicated framework for the rational development of selective NMDAR based drug therapies for AD. Investigations at the receptor subunit level may be particularly useful in attempts to identify unique NMDAR subtypes that are selectively affected in AD

APP AND RELATED PROTEINS

The term APP designates a complex group of type I integral membrane glycoproteins, 100 – 140 kDa in relative molecular mass with a single transmembrane domain, that undergoes N- and O- linked glycosylation, phosphorylation, sulphation and proteolytic cleavage (Oltersdorf et al., 1990; Schubert et al., 1989b; Selkoe et al., 1988; Weidemann et al., 1989). Heterogeneity of APP molecules arises from alternative splicing of a single gene, which in humans is located on the long arm of chromosome 21. The major form of APP in the brain exsists as a 695 amino acid protein (APP695) associated predominately with neurons, while minor isoforms including APP717, APP751 and APP770 tend to be associated with glial cells (Mattson et al., 1993; Mucke et al., 1995; Mucke et al., 1994). Although APP is expressed in virtually all mammalian cells and has been the subject of intensive investigation for over a decade, a unifying concept regarding a biological role for this protein is still to emerge. A considerable body of data has indicated a role for APP in promoting neuronal survival. Exogenously added APP or C-terminally truncated fragments of APP, normally secreted from the cell surface during proteolytic processing, can protect primary neuronal cultures or cell lines from a variety toxic insults including hypoglycemia, glutamate excitotoxicity or Aβ toxicity (Goodman et al., 1994a; Mattson et al., 1993a; Schubert et al., 1989b). APP appears to exert its protective effects by stabilizing intracellular calcium ($[Ca^{2+}]_i$) levels (Mattson et al., 1993a).

APP is proteolytically processed by at least three secretase enzymes along two distinct metabolic pathways (fig. 1.1; for reviews see Hardy, 1997; Nunan et al., 2000; Selkoe, 1994c; Selkoe, 1994a; Selkoe et al., 1988). The APP molecule is initially cleaved by either α-secretase within the β-amyloid sequence or by β-secretase at the NH_2 end of the β-amyloid sequence resulting in the secretion of a soluble N-terminal fragment of APP (sAPP) from the cell surface (Anderson et al., 1991; Haass et al., 1992).

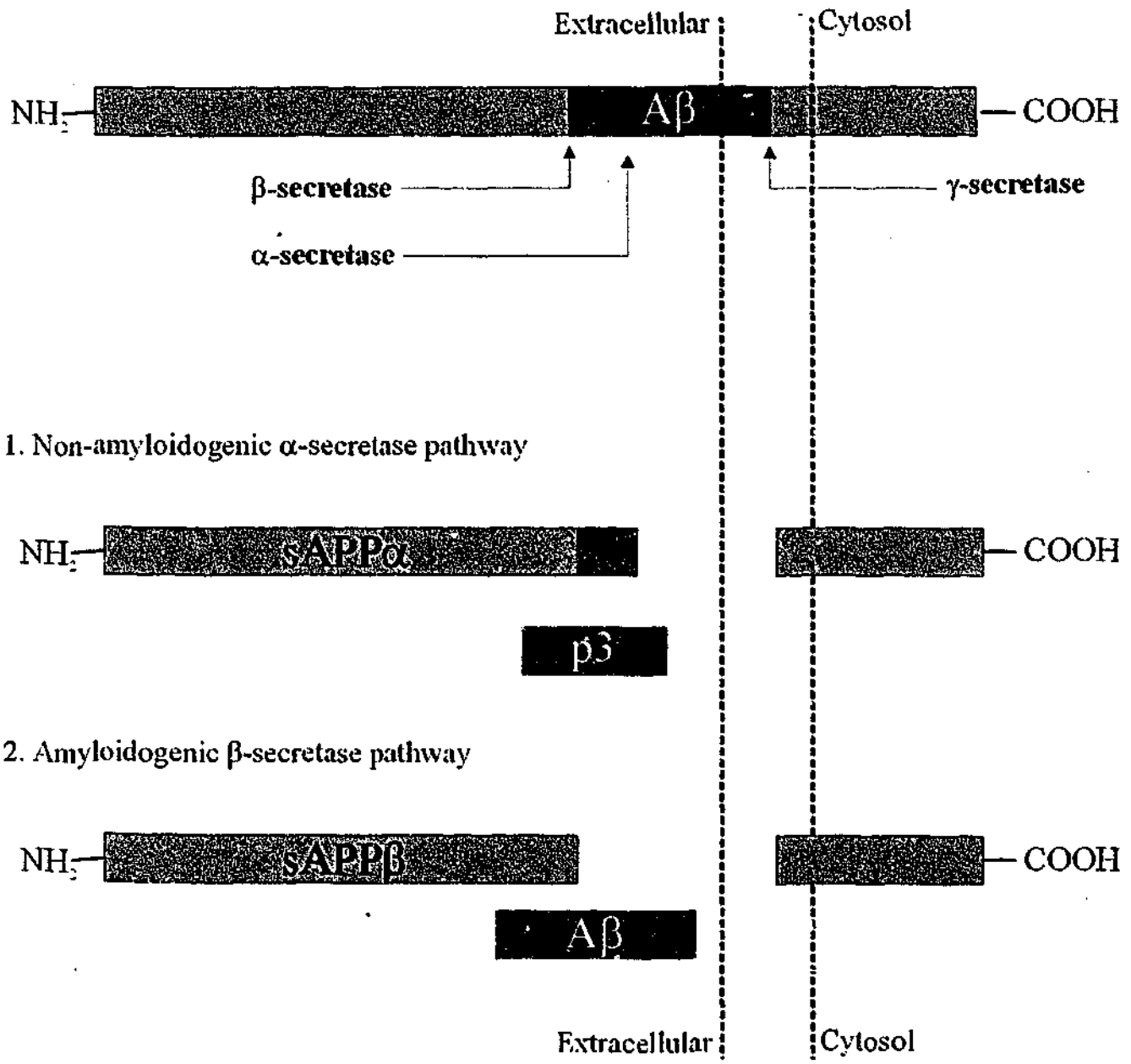


Figure 1.1. The non-amyloidogenic and amyloidogenic proteolytic APP processing pathways mediated by α-secretase, β-secretase and γ-secretase. Abbreviations: Aβ, β-amyloid; sAPPα, secreted APP-α; sAPPβ, secreted APP-β.A recent study has reported that α and β-secretase processing may be competitive in nature whereby processing by one secretase precludes the action of the other (Skovronsky et al., 2000). Following α/β-secretase processing, the remaining membrane bound C-terminal fragment is cleaved within the lipid membrane by γ-secretase (Seubert et al., 1992).

Non-amyloidogenic processing of APP occurs via the α pathway following α-secretase mediated cleavage within the β-amyloid sequence, which results in the secretion of the N-terminal fragment of APP (sAPPα) from the cell surface, and subsequent cleavage by γ-secretase, which liberates a fragment of the β-amyloid sequence ($A\beta_{(17-42)}$) termed 'p3'. The physiological significance of p3 is not well understood at this stage. The amyloidogenic β pathway is mediated by β and γ-secretase cleavage, which results in the secretion of the N-terminal fragment of APP (sAPPβ) and liberation of the β-amyloid peptide sequence. Following α/β-secretase cleavage, the exact cleavage site of the remaining C-terminal fragment may vary, resulting primarily in the formation of a relatively soluble 40 amino acid β-amyloid peptide ($A\beta_{(1-40)}$) but also the longer 42 amino acid isoform ($A\beta_{(1-42)}$). It is not clear whether this is due to poor specificity of γ-secretase at the cleavage site or the activity of multiple enzymes. Under normal physiological conditions, the longer $A\beta_{(1-42)}$ peptide typically accounts for less than 10% of total Aβ production but up to 25% of Aβ produced in neuronal cells (Tienari et al., 1997; Turner et al., 1996). $A\beta_{(1-42)}$ is more hydrophobic and considerably less soluble then $A\beta_{(1-40)}$ and readily forms Aβ fibrils *in vitro* via a nucleation dependent mechanism (Jarrett et al., 1993). Despite $A\beta_{(1-42)}$ representing a small fraction of Aβ production, this is the major isoform found in neuritic plaques (Fukumoto et al., 1996; Roher et al., 1993) and its accumulation is thought to act as a template for the fibrillation of otherwise soluble $A\beta_{(1-40)}$ (Jarrett et al., 1993).

The identification of the α, β and γ-secretase proteins is an area that is presently receiving a great deal of attention in relation to AD research. The γ-secretase protein has been the subject of most of this attention and consequently its activity is well characterized. The two most likely candidates for the role of γ-secretase are presenilin-1 (PS1) and presenilin-2 (PS2). Presenilins (PSs) are integral membrane proteins with eight

putative transmembrane domains, encoded by genes on chromosomes 14 (PS1) and 1 (PS2) (Nishimura et al., 1999). A number of mutations have now been identified in PSs (predominately PS1) which are associated with FAD and many of these mutations result in an increase in the $A\beta_{(1-42)}/A\beta_{(1-40)}$ ratio (Kovacs et al., 1998; Scheuner et al., 1996). There is a good deal of evidence to suggest that PSs are in fact γ-secretases. Certainly PSs are required for γ-secretase activity as demonstrated by the finding that knockout of both PS1 and PS2 completely abolishes γ-secretase activity (Herreman et al., 2000). Furthermore, both PSs are localized to the site of γ-secretase processing (endoplasmic reticulum/Golgi apparatus) (Kovacs et al., 1996) and form stable complexes with APP (Weidemann et al., 1997; Xia et al., 1997). It should be noted however that the PSs have never been shown to possess protease activity, which casts some doubt over their identity as γ-secretases. The activities of α and β-secretase have also been extensively studied in relation to AD research (for review see Racchi et al., 1999). One of the important features associated with processing of APP by α-secretase is that the cleavage site is within the Aβ sequence and so it is generally thought that the α-secretase pathway mitigates Aβ formation. If indeed α and β-secretase compete for the same cellular machinery as has been suggested (Skovronsky et al., 2000), stimulation of the α-secretase pathway may prevent the amount of Aβ formed during APP processing by reducing the relative amount of amyloidogenic β-secretase processing. Another important feature of α-secretase processing is that the resultantly secreted N-terminal fragment, sAPPα, may have trophic and neuroprotective activities (Mattson et al., 1993b; Small, 1998). Although the identity of the α-secretase protein is not currently known, a likely candidate is a member of the ADAM (a disintegrin and metalloprotease) family called tumor necrosis factor-α converting enzyme (TACE). TACE has been shown to cut APP at the α-secretase cleavage site *in vitro*, and inhibitors of TACE block the stimulated secretion of sAPPα from cells (Black et al., 1997; Buxbaum

et al., 1998; Moss et al., 1997). Furthermore, fibroblasts from TACE knockout mice generate lower levels of sAPPα (Buxbaum et al., 1998). The β-secretase APP cleaving protein (BACE) has been identified by several groups following genetic screening and direct enzyme purification and sequencing (Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999a; Vassar et al., 1999; Yan et al., 1999). BACE is a member of the pepsin family of aspartyl proteases that is expressed initially as a preproprotein and then efficiently processed to its mature form in the Golgi (Haniu et al., 2000). A related transmembrane aspartyl protease (BACE 2) shows similar substrate specificity (Farzan et al., 2000; Yan et al., 1999) but is not highly expressed in the brain (Bennett et al., 2000). BACE is expressed coordinately with APP in many regions of the brain, particularly in neurons, and has a subcellular distribution similar to that of β-secretase (Vassar et al., 1999). Regardless of whether the PSs, TACE and BACE actually represent the secretases that cleave APP or alternatively, act indirectly as cofactors, the critical involvement of these proteins in APP processing and subsequent production of physiologically important molecules such as Aβ and sAPPα makes them interesting targets in AD research.

Given the important relationship between Aβ accumulation and AD, changes in the expression and processing of APP are certainly likely to be related to the disease process. A significant amount of evidence has accumulated over the last decade implicating APP in the pathogenesis of AD, including reports that: mutations in the APP gene are associated with early onset forms of the disease (Clark et al., 1993; Goate et al., 1991); over-expression of mutated APP in transgenic mice results in AD-like pathology (Games et al., 1995); APP degradation products accumulate in brains of patients with AD (Sisodia et al., 1990); and that APP abnormally accumulates in neuritic plaques and in synaptic terminals (Arai et al., 1991; Cole et al., 1989; Cras et al., 1991; Masliah, 1995; Masliah et al., 1992). Much of the research has focused on investigating how APP and its metabolism might be involved in the pathogenesis of the disease rather then looking at how the

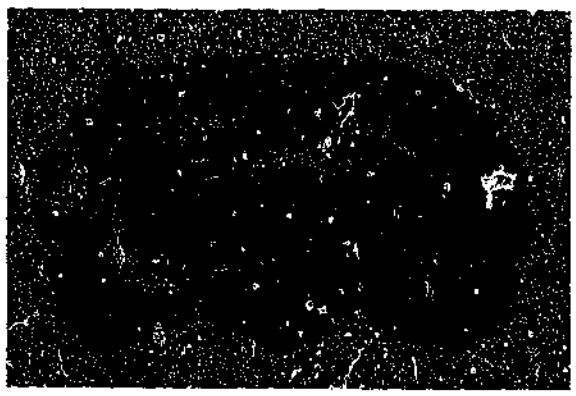
neurodegenerative processes in AD affect APP and the secretase enzymes which process it. One of the aims of this project has been to investigate how rat models of specific aspects of AD, such as degeneration of the basal forebrain nuclei and accumulation of Aβ, might affect the mRNA expression of APP695 and a number of the proteins which have been shown to have important roles in its metabolism including PS1, PS2, TACE and BACE. The effect of transient knockdown of APP695 on other neurochemical markers that may be important in AD is also an area that has been investigated here.

Following decades of intensive research that has established AD as the most widely studied neurodegenerative disorder to date, a number of theories have emerged which attempt to characterize the etiology of AD in terms of a single, unifying event that underlies the neurodegenerative process. The two most prominent theories are the amyloid cascade hypothesis (for review see Hardy, 1997) and the cholinergic hypothesis (for review see Bartus, 2000), which suggest primary and causative roles for Aβ accumulation and cholinergic dysfunction respectively in the disease process as will be discussed in the following chapters. Although there is little doubt that Aβ accumulation and cholinergic function are critically important factors associated with the disease, it is likely that these events are necessary but not sufficient for the pathogenesis of AD. Indeed, most researchers would argue that the etiology of the disease is likely to be characterised by the persistence of the combination of a number of specific genetic and environmental factors over extended periods of time. Unlike many other neurodegenerative conditions, such as Parkinson's disease, which are characterised primarily by the dysfunction of a single neuronal system, these factors lead to the dysfunction and degeneration of multiple neuronal systems in AD. Consequently, it seems likely that preventative and particularly palliative treatment of the disease will involve a combination of therapeutic approaches. The central aim of this thesis has been to explore the relationship between specific aspects of the neurodegenerative and morphological changes seen in AD and the function and

expression of a variety of neurochemical markers that may contribute to the neurodegenerative process and/or represent therapeutic targets. Based on the selective vulnerability of the cortex and hippocampus to the neurodegenerative changes seen in AD, and the importance of these structures in the process of learning and memory, the work presented throughout this thesis has focused specifically on the quantification of these neurochemical markers in various hippocampal and cortical regions. Establishing the nature of the relationship between the multitude of neurochemical changes see in AD, particularly in terms of cause and effect, is something that still largely lies ahead of AD researchers and will be an important part of gaining a better understanding of the pathogenesis of the disease.

Chapter 2

General Methodology



A number of techniques which are have been used repeatedly throughout this body of work are described here in detail and are readdressed briefly in the relevant chapters. Unless otherwise stated, all reagents were obtained through either Sigma Aldrich or RBI.

Phosphate buffered saline (PBS) was often used in surgical procedures. A 10 x PBS solution was prepared as 1.0 M NaCl, 70 mM anhydrous Na_2PO_4 and 30 mM $Na_2PO_4.H_20$ (pH 7.4).

Glass slides used for tissue mounting were baked at 200°C for 4 hours to eliminate any RNAase activity. Each slide was dipped in a solution of 0.01% (v/v) poly-L-lysine/ diethyl pyrocarbonate (DEPC)[1] -treated dH_20, air dried and stored at -20 °C.

2.1 In Situ Hybridisation Histochemistry

The technique of *in situ* hybridisation histochemistry (ISHH) has been used extensively throughout this study to demonstrate the anatomical distribution and relative levels of expression of various mRNA species. The end product of an ISSH experiment is a radiographic image depicting mRNA expression at the level of the cell body in a given tissue sample. The protocol used is essentially based on the methods described by Wisden et al., (1994) and updated by Loiacono et al., (1999).

Fixing and Delipidation

Fresh, frozen, slide mounted tissue sections were allowed to come to room temperature before being dehydrated in graded ethanol (EtOH)/DEPC-dH_20 solutions and treated with chloroform as follows: 5 min 70% EtOH, 2 min 95% EtOH, 2 min 100%

[1] DEPC treated dH_20 (1ml/L) was used in place of regular dH_20 when making solutions to eliminate the presence of biological contaminants such as RNAse.

EtOH, 10 min chloroform, rinse 100% EtOH. Following the last EtOH rinse the sections were either stored in absolute EtOH at 4°C or air dried for immediate use. This process delipidates the tissue sections, which significantly reduces background noise and fixes cell membranes thereby effectively trapping mRNA within the cells and thus ensuring it remains tissue bound during the course of the experiment. In comparison to the more common 10% paraformaldehyde fixation method, the use of fresh, frozen sections delipidated in chloroform enables better presentation of tissue mRNA at the expense of a slight reduction in the preservation of tissue morphology.

Preparation of Oligonucleotide Probes

Synthetic oligonucleotide probes complementary to selected base sequences within the full mRNA sequence encoding for the various proteins of interest were synthesised by Life Technologies (Melbourne, Australia). All probe sequences used have been listed in table 2.0.

All oligonucleotides were constructed of 45 bases with no modifications. The sequences were checked for cross-recognition in a BLAST search and can be retrieved from Genbank using the specified accession numbers. As a number of splice variants are known to exist for the α_4 nAChR subunit, oligonucleotide probes were designed to target regions common between the variants. The probes targeting the α_4 subunit are therefore 'pan' probes and do will not distinguish between different α_4 subunit mRNA splice variants.

Table 2.0. Sequences and accession details for the oligonucleotides used for mRNA detection in ISHH experiments.

Target	Accession #	Sequence of oligonucleotide	Reference
α3	L31621	5'-TTCGAACAGGTACTGGAACAGGCGGTGCTCAGCTTCTGAGGCACT-3' 5'-GACCCAAGTGGGCATGGTGTGTGTGGTTGGAGTTCTATAGTGCAC-3' 5'-ATCTTGGCAGGGGTAGCCTTCCTTGCAGCTTTTGGAGTCTGCACG-3'	(Boulter et al., 1987)
α4	L31620	5'-ACATGCTGGACACTCAGGGACCTGGCTTTGATGAGCATTGGAGCC-3' 5'-CTGGGGACACAGGAGATGGTTCCTTGCATGTGCATTTGCATGGAG-3' 5'-TGTCTTCTGCCTTGAGGTGGTCTGCAATGTACTGGACGCCTTCTA-3'	(Goldman et al., 1987)
α5	J05231	5'-ATCTTACGAACCCAGGGCGCCATAGCGTTGTGTGTGGAGGAAGAG-3' 5'-TTGGTAGGGTTTGCCAGGATGCAGATGCTAGGCCTTCACTAGTGC-3' 5'-GCACACCCATGGTCCCAGCTACTCAGGAGGTTTTGTGCTAGCCCA-3'	(Boulter et al., 1990)
α6	L08227	5'-TCAAAGTGCACCGTGACGGGATCAGAAACGTTTTCCACTGGCCGG-3' 5'-AGGAACATGGTCTTCACCCACTTGGGCATGGTATGCGTTGCTGGG-3' 5'-GGTGGGTGCTCTGAATTCTCTGTTACCCACTGTGCAGGCTGCTGG-3'	Unpublished.
α7	S53987	5'-ACAAGGGATGAGCAGATTGAGGCCATAGTAGAGTGTCCTACGGCC-3' 5'-GTCCTCTCCGGGCCTCTTCATGCGCAGAAACCATGCACACCAGTT-3' 5'-TGCCCTCCAGGCCTCGGAAGCCAATGTAGAGCAGGTTGCCATTGC-3'	(Seguela et al., 1993)
β2	L31622	5'-AGCCAAGCCCTGCACTGATGCAGGGTTGACAAAGCAGGTACATGG-3' 5'-TCGCATGTGGTCCGCAATGAAGCGTACGCCATCCACTGCTTCCCG-3' 5'-AGGCTGCAGGAACATGCCGACGGTCCCAAAGACACAGACAAAGAC-3'	(Boulter et al., 1987)
β3	J04636	5'-AGGGGGCCATGGGATGGTACGTTGAGGAAGATCTGTGGTGGACAT-3' 5'-TCGGAGGCCTTCTCGAGGAAAGCGACCAGAACTCTTTCTCCATCG-3' 5'-TACGATTGCATGGCTCTGCTTCCTGTCAGCATGCCAGCCGCTGTC-3'	(Deneris et al., 1989)
APP695	AY011335	5'-GCTGGCTGCCGTCGTGGGAACTCGGACCTACCTCCTCCACAGACTC-3'	(Murphy et al., 2001)
PS1	D83948	5'-TGGCCACCACAACGACCATGCAGAGGGTCACAGGAACAAAGAGCA-3' 5'-AGGGCACTGATCATAATGAGATACGCCTGCTGCAGTCGGAGTGGG-3' 5'-CTCCCAGTGCCAGTGTCCTGTGTCTCTTCCCTCTCTGTTCCTTGT-3'	(Takahashi et al., 1996)
PS2	AB004454	5'-AACGTGATGTAGGGCTCTCGGCTGACATCAAGGACGTCCGCTCAT-3' 5'-TGGCTGCTGCCTTGCCCACCAGAACGCTGTAGAAGATGAAGTCTC-3' 5'-GGAGCATCAGGGAGGACATGATCAGCCAGCCATGGATGAACTTGT-3'	(Tanahashi et al., 1998)
BACE	AF190727	5'-TGGCCGGACTTTCCCCTCAGGTTGTCCACCATCTCCACAAAGCTG-3' 5'-GTGGGTCTGCTTCACCAGGGAGTCAAAAAAGGGCTCCAAGGAGTC-3' 5'-CAAAAGCCATCCGGGAACTTCTCCGTCGAGGAGGCTGCCTTGATG-3'	(Vassar et al., 1999)
TACE	AJ012603	5'-GAGAAACTGCTCACATTGGGAAGCGGAGGTGATCTTCCCAGCACG-3' 5'-AGGTTCAGCTCGCCTCTTCACTCGACGGACAAACTCTTCAGATGG-3' 5'-CTCTGCATCGACGTAAGGCACACATGGGCCAGAAAGGTTCCTGCA-3'	Unpublished.
NR1/2A/2B		Provided by H. Monyer, University of Hiedelberg, Germany.	Personal communication
nNOS	X59949	5'-TCACCAGGAAGCCCAGACCTCCCACTTTGCGTTTGAAGAGACGAA-3' 5'-TTCATCATGTTCCCCGATGTCCTGGAGGTTGGCCTTGGTGCTTTT-3'	(Bredt et al., 1991)

The probes were diluted in DEPC-dH_2O to a working stock concentration of 3 ng/ml and stored at -20°C. Each probe was radiolabelled at the 3' end with a σ - ^{33}P deoxyadenosine 5' - triphosphate ([^{33}P]α-dATP; 3000 Ci/mmol; NEN™ Life Science Products Inc., USA) nucleotide tail using a standard terminal transferase labelling kit (Boehringer Mannheim, Australia). The reaction was carried out at 37°C and terminated by the addition of 38 µl DEPC-dH_2O. This solution was then added to pre-spun Sephadex columns[2] and centrifuged at 2000 rpm for 2 min in order to separate unincorporated tracers from the radiolabelled probes. A 1 µl sample of each probe was assessed for the extent of specific labelling using a Packard TriCarb scintillation counter. Counts for successfully labelled probes were generally between 1.0×10^5 and 3.0×10^5 dpm/µl.

As indicated in table 2.0, up to three separate probes were synthesised for the detection of each individual mRNA target due to the relatively low levels of expression in the brain. A spacing of at least 100 base pairs was left between the end of one probe and the beginning of the next, so that the addition of the radiolabelled tail would not restrict the access of other probes to the targeted mRNA sequence during the hybridisation process.

Hybridisation

For each mRNA target, the corresponding probe/s were diluted 50-fold in a minimalist hybridisation buffer solution and vortexed for 5 min. (i.e. to make the buffered solution for the α_7 nAChR subunit, 50 µl of each of the three separately radiolabelled oligonucleotides were added to a final volume of 2.5 ml of the hybridisation buffer). Slide mounted sections were air dried, placed in sterile petri dishes, covered with 100 µl each of the appropriate buffered probe solution and covered with a Parafilm™ slip. A small wad of

[2] Spin solution was prepared as 14 ml 5 M NaCl, 10 ml 1 M Tris (pH 8.0), 5 ml sodium dodecyl sulphate, 466 ml DEPC-dH_2O and 20 g Sephadex G-25™ (Pharmacia, Australia). The solution was autoclaved and stored at 4 °C. Spin columns were constructed by placing a small wad of glass wool at the base of 1 ml syringes, filling the remaining volume with spin solution and centrifuging at 2000 rpm for 2 min.

tissue paper soaked in a 50% v/v solution of formamide in 5x sodium citrate/sodium chloride (SSC; pH 7.0)[3] was added to the petri dish to prevent drying of the tissue sections. The petri dishes were covered and placed in an oven at 42 °C for 16 hours during which time hybridisation of the labelled probes to the tissue bound mRNA took place.

Washing

Following hybridisation the Parafilm™ slips were removed and slides were rinsed briefly in a room temperature solution of SSC, transferred to SSC at 60°C for 1 hour, rinsed in SSC then 0.1x SSC and finally dehydrated in graded solutions of ethanol/DEPC-water. After drying, the slide-mounted sections were apposed to β-max Hyperfilm (Amersham, USA) in light-safe cassettes (24 x 30 cm; Planex, Australia) for various periods of time, depending on the mRNA being examined. Exposure times were as follows:

- APP695: 72 hours
- NMDAR NR1 subunit: 10 days
- nAChR α_4 subunit: 2 weeks.
- nAChR α3, α5, α6, α7, β2 & β3 subunits; NMDAR 2A & 2B subunits; BACE; PS1: 3 weeks.
- TACE; PS2; nNOS: 5 weeks.

Films were processed at room temperature in light-safe conditions (Ilford 906 grade red-light filter, Australia) as follows: 5 min in a solution of Phenisol X-ray film developer (Ilford, Austalia), rinsed in a mixture of water and stopbath (Ilford, Austalia) for 1 min and fixed in hyperfilm rapid-fixer (Ilford, Austalia) for 10 min.

[3] 20x stock is 3 M NaCl and 3 M sodium citrate in DEPC-dH_2O

2.2 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY

The protocol for [^{3}H]epibatidine autoradiography was based on that described by Perry et al., (1995b). Slide mounted sections were incubated with 0.46 nM [^{3}H]epibatidine (66.60 Ci/mmol; Dupont, USA) at room temperature for 40 min in an incubation buffer consisting of 50 mM Tris-HCl (pH 7.0), 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$. Non specific binding was determined by the addition of 300 µM (-)-nicotine in adjacent sections. Following incubation, the slides were taken through 2 x 5 min rinses in ice-cold buffer. The sections were then dried immediately with a stream of air and apposed to tritium-sensitive film (Hypermax Hyperfilm; Amersham, USA) in light-safe cassettes for 12 weeks. A set of standard tritium scales (Amersham, USA) were also apposed to film for each experiment. Films were developed at room temperature for 5 min, rinsed in water and stopbath for 1 min and fixed for 10 min, as previously described.

2.3 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY

The protocol for [^{125}I]α-BGT was based on that used by Whiteaker et al., (1999). Slide mounted sections were pre-incubated in binding buffer consisting of 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 200 mM Tris-HCl, 20 mM HEPES and 0.1% (w/v) bovine serum albumin (pH 7.5) for 20 min at room temperature. Slides were then incubated with 2 nM [^{125}I]Tyr^{54}-α-Bungarotoxin ([^{125}I]α-BGT; 152 Ci/mmol; NEN™ Life Science Products Inc., USA) in binding buffer for 4 h at room temperature. Non-specific binding was determined by incubating adjacent sections from each animal in the same conditions but with the addition of 10 mM (-)-nicotine in the binding buffer. All washing was done at 0°C and consisted of: 2 x 10 min in binding buffer, 2 x 5 s in 0.1x binding buffer and 2 x 5 s in 5 mM HEPES (pH 7.5). Slides were dried with a stream of air

immediately after washing and apposed to β-max Hyperfilm (Amersham, USA) for 5 days. Films were developed at room temperature for 5 min, rinsed in water and stopbath for 1 min and fixed for 10 min, as previously described.

2.4 AChE Histochemistry

AChE levels were quantified using a modification of the method previously described by Koelle et al., (1949). Slide mounted sections were incubated for 45 min at 37°C in an incubation medium of the following composition: 150 mg acetylthiocholine, 195 ml acetate buffer[4] (pH 6.0), 15 ml 0.1 M sodium citrate, 30 ml 30 mM copper sulphate, 30 ml dH_2O and 30 ml 5 mM potassium ferricyanide. Following incubation, slide mounted sections were rinsed in dH_2O, dehydrated in graded ethanol/dH_2O solutions (rinsed in 70% EtOH, 95% EtOH, 100% EtOH), treated with xylene and cover-slipped for analysis.

2.5 Quantitation

Images generated through the ISHH and receptor binding protocols were digitally captured on PC under a windows 98 operating system using a COHU high performance CDD camera and graphical interface card (USA) and a Nikon lens (Micro-NIKKOR, 55mm, 1:2.8, Japan). A Biotec-Fischer lightbox (Germany) was used as the lightsource underneath the images. The optical density of selected anatomical regions was quantified using Scion Image software (www.scioncorp.com). For mRNA images, [^{125}I]α-BGT

[4] 50 mM sodium acetate adjusted to pH 6.0 with 50 mM acetic acid.

autoradiographs, and histochemical AChE stains the system was calibrated against a set of graded greyscales (Kodak; fig. 1), allowing for quantitation in terms of relative optical density (ROD). For tritium applications, including [^{3}H]epibatidine and [^{3}H]hemicholinium autoradiographs, the system was calibrated against a set of standard tritium scales (fig 2.0), allowing for quantitation in terms of nCi/mm^2. Tritium scales were generated by apposing a set of standard tritium scales (Amersham, USA), with a known tritium content in nCi/mm^2, to tritium sensitive film for the same time as the slide mounted tissue.

Tritium scale **Kodak greyscale**

Figure 2.0. Tritium and grey scales were used to calibrate the Scion imaging software for measurement of the optical density of specific anatomical regions in the radiographic or histological images. The radioactivity associated with each gradation in the tritium scale, in nCi/mm^2, is as follows: (a) 1.4, (b) 2.2, (c) 3.3, (d) 5.5, (e) 8.8, (f) 13.1, (g) 21.2 and (h) 33.5. The arbitrary ROD units assigned to the Kodak greyscale were as follows: (i) 0.1, (ii) 0.2, (iii) 0.3, (iv) 0.4, (v) 0.5, (vi) 0.6, (vii) 0.7, (viii) 0.8, (ix) 0.9 and (x) 1.0.

Using the Scion imaging software, it was possible to generate calibration curves of optical density against either nCi/mm^2 using the tritium scale, or ROD using the Kodak scale. Based on the relationship between tritium content in the tritium scales and optical density, a log-log linear curve fit was used. A linear curve fit was used for the optical dentity/ROD data. Measurements of optical density for radiographic and histological images were expressed in terms of either nCi/mm^2 or ROD through interpolation or extrapolation of the appropriate calibration curve.

Every effort was made to minimise artefactual variation in optical density between control and treatment groups in a given experiment. Factors that may introduce such

variation into experiments where the results are measured in terms of optical density include:

- Film sensitivity, film exposure time, film processing parameters.
- Efficacy of probe radiolabelling, age and specific activity of the radionucleotide.
- Lightbox intensity, ambient lighting when capturing images.
- Lens aperture and focal length.

These factors were not controlled for, in regard to sets of data not intended for statistical comparison. Consequently, the ROD measurement corresponding to, for example a certain mRNA, may vary in different sets of control tissue from different experiments. However, it has not been the intention of the work presented here to comment on the measurement of neurochemical markers in naïve animals. Rather, the intention has been specifically to compare differences in the measurement of neurochemical markers between control and treated tissue in a given experiment. In this context, factors that may introduce artefactual variation were strictly controlled for. Groups of control and treated tissue sections intended for direct statistical comparison were always exposed to the same sheet of film and digitally captured under the same conditions in terms of lighting and lens specifications. This ensured that any differences in optical density between control and treatment groups was due to experimental manipulation and not artefactual variation.

ROD or nCi/mm^2 measurements for all treatment groups were always taken from the left hemisphere. No assessment has been made regarding possible bilateral effects associated with experimental manipulation. Differences in ROD or nCi/mm^2 between control and treatment groups were assessed statistically using either one-way analysis of variance (ANOVA) with the appropriate post-test, or an unpaired t test, depending on the number of treatment groups. These statisitical tests were always performed on the mean value of measurements taken from at least 4 animals. Where possible the average of duplicate measurements taken from each animal was used. P values of less then 0.5 have

been taken to signify statistical significance throughout this thesis. The statistical tests used for each experiment have been discussed further in each chapter.

A schematic of a coronal section of rat brain 3.6 mm caudal to bregma, showing some of the anatomical regions commonly analysed in this study has been presented below in figure 2.1. This figure is particularly useful for illustrating the distribution of cortical layering.

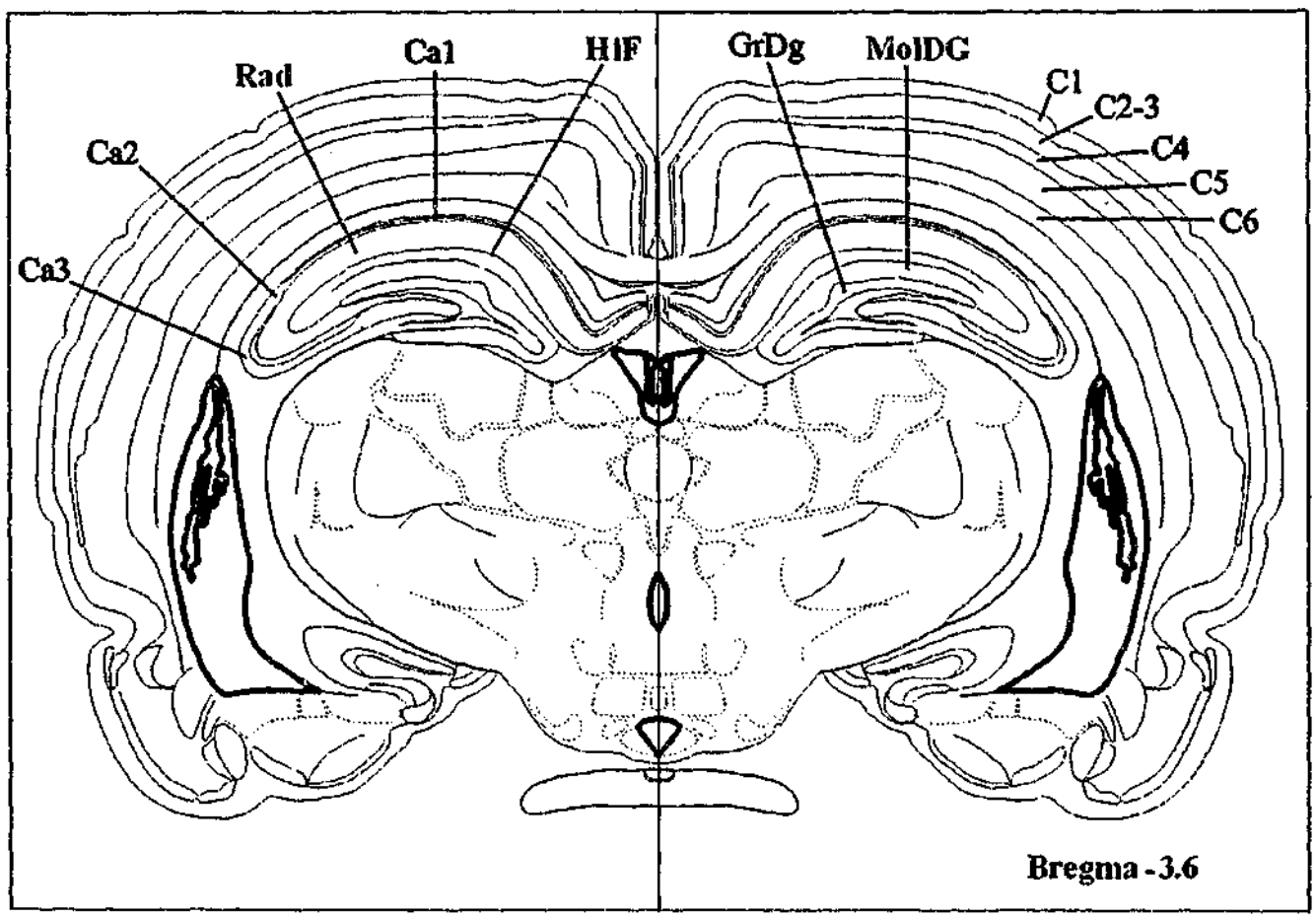


Figure 2.1. Schematic representation of a coronal section of rat brain 3.6 mm caudal to bregma, adapted from the atlas of Paxinos and Watson (1986). Anatomical regions commonly analysed throughout the work presented here have been labelled. Abbreviations: Ca1 – 3, fields of the hippocampus; C1, cortical layer 1; C2-3, cortical layers 2 – 3; C4, cortical layer 4; C5, cortical layer 5; C6, cortical layer 6; GrDG, granular layer of the dentate gyrus; HiF, hippocampal fissure; MolDG, molecular layer of the dentate gyrus; Rad, stratum radiatum hippocampus.

Chapter 3

The effect of *in vivo* β-amyloid & chronic nicotine treatment on multiple neurochemical markers in the rat brain

3.1 INTRODUCTION

Describing the neuropathology of his first reported case in 1907, Alois Alzheimer wrote:

"*Scattered through the entire cortex, especially in the upper layers, one found miliary foci that were caused by the deposition of a peculiar substance in the cerebral cortex*" (Alzheimer, 1907).

This 'peculiar substance' has since been identified as the β-amyloid peptide and is the major constituent of the neuritic plaques that histopathologically characterise the disease (Glenner et al., 1984; Masters et al., 1985). Following almost a century of research since Alois Alzheimer's initial findings, there is still some debate as to whether or not the Aβ peptide plays a causative role in the pathogenesis of AD. Certainly, there has been some compelling evidence to emerge from this research that implicates the involvement of Aβ in the disease process on some level. For instance, cerebral Aβ deposition is an invariant feature of AD, and the fibrogenic $A\beta_{(1-42)}$ isoform which is intimately associated with dystrophic dendrites, axons, microglia and astrocytes in the form of neuritic plaques (Dickson, 1997; Terry et al., 1994) is substantially more abundant in the limbic and association cortices of AD brains than in those from age-matched controls in virtually all cases. Perhaps the most important finding which supports a role for Aβ in AD pathogenesis relates to the relationship that has been shown to exist between genetic mutations that affect the metabolism of APP and the incidence of early onset or familial

forms of the disease (table 3.0). Various mutations in either the APP, PS1 or PS2 genes are associated with clinical and pathological features of AD and cause either a significant increase in the production of Aβ or a shift in the metabolism of APP which favours the production of $A\beta_{(1-42)}$ over $A\beta_{(1-40)}$. Transgenic mice that over-express mutated forms of APP exhibit certain aspects of AD pathology (Games et al., 1995). Furthermore, individuals with Down's syndrome, who possess an extra copy of the APP gene, develop amyloid plaque like morphology and exhibit certain clinical symptoms associated with AD such as dementia (Giaccone et al., 1989; Mann et al., 1989; Mann et al., 1986; Motte et al., 1989; Wisniewski et al., 1985). A useful review of the genetics of AD has been published by Lendon et al., (1997).

Gene	Mechanism	Biochemical Effect	Author
APP (Down's syndrome)	Extra copy of the APP gene (chromosome 21 trisomy).	Increase in total Aβ production.	(Wisniewski et al., 1985)
APP_{716} (Florida)	Mutation at γ-secretase cleavage site.	Increase in the ratio of $A\beta_{(1-42)}/A\beta_{(1-40)}$.	(Eckman et al., 1997)
APP_{717} (London)	Mutation at γ-secretase cleavage site.	Increase in the ratio of $A\beta_{(1-42)}/A\beta_{(1-40)}$.	(Goate et al., 1991; Suzuki et al., 1994)
$APP_{670/671}$ (Swedish)	Mutation at β-secretase cleavage site.	Increase in total Aβ production.	(Cai et al., 1993; Citron et al., 1992; Mullan et al., 1992)
APP_{693} (Dutch)	Mutation at α-secretase cleavage site.	Vascular amyloid deposition, cerebral haemorrhage.	(Levy et al., 1990)
APP_{692} (Flemish)	Mutation at α-secretase cleavage site.	Cerebral haemorrhage, reduced p3 formation.	(Hendriks et al., 1992)
PS1/PS2	Missense mutations cause altered processing at γ-secretase cleavage site.	Increase in the ratio of $A\beta_{(1-42)}/A\beta_{(1-40)}$.	(Borchelt et al., 1996; Sherrington et al., 1995; Tomita et al., 1997)

Table 3.0. Genetic mutations which cause β-amyloid deposition and plaque formation and associated with the incidence of early onset familial AD. Abbreviations: Aβ, β-amyloid; APP, amyloid precursor protein; PS1/2, presenilin-1/2.

The aforementioned data along with the lack of evidence for a specific defect in metabolism, loss of trophic factor, or viral or toxic agent other then Aβ that can be said to be etiologic for AD, has lead to the emergence of the 'amyloid cascade hypothesis'. The general form of this hypothesis predicts that all causes of AD should have in common the feature that they make Aβ deposition more likely, and that the accumulation of Aβ is the primary event underlying the pathogenesis of the disease (Hardy, 1997). While there is a good deal of evidence to support such an idea, a considerable amount of work still needs to be done before the amyloid cascade hypothesis can be validated as an accurate description of the causative, pathogenic mechanism underlying AD. Much of this work will involve exploring the relationship between Aβ and the progressive neurodegeneration seen in AD.

A pervading assumption among supporters of the amyloid cascade hypothesis is that Aβ is toxic to nerve cells. This has been somewhat of a contentious issue in AD research and there have been numerous studies that have both supported and refuted this assumption. On balance, it seems that Aβ peptides are likely to possess neurotoxic properties *in vitro* which are dependent on both the concentration and tertiary conformation of the peptide (Mattson et al., 1992; Pike et al., 1993; Yanker et al., 1990). More aggregated forms of the peptide, such as $A\beta_{(1-42)}$, have been shown to exhibit greater neurotoxicity in neuronal cell culture systems compared with the more soluble $A\beta_{(1-40)}$ form of the peptide (Pike et al., 1993). Interestingly, while neurotoxic concentrations of Aβ peptides have typically been in the order of 1 – 100 μg/ml, low concentrations of Aβ have been reported to have neuroprotective properties *in vitro* (Whiston et al., 1989; Yanker et al., 1990). The results in regard to the neurotoxicity of Aβ peptides *in vivo* have generally been much less consistent then those generated from *in vitro* studies (table 3.1). Transgenic mice that show extensive deposition of amyloid in the form of plaques and increased serum levels of $A\beta_{(1-42)}$ do not display concomitant neuronal loss (Games et al., 1992; Hsiao et al., 1996). Studies that involve the injection of synthetic Aβ peptides directly into the

brains of rats have reported either neurotoxicity and/or associated behavioural deficits (e.g. Giovannelli et al., 1995; Harkany et al., 1995; Nitta et al., 1997) or a lack of neurotoxic effect or any discernable AD-like pathology (e.g. Abe et al., 1994; Winkler et al., 1994). Clearly, there are a number of physiologically relevant factors present during *in vivo* studies, such as glial-neuron interactions and the influence of many transmitter systems, which do not affect results generated from *in vitro* paradigms. While $A\beta_{(1-42)}$ has been shown to be the more neurotoxic form of Aβ *in vitro*, a study by Shin et al., (1997) has reported that injections of $A\beta_{(1-40)}$ but not $A\beta_{(1-42)}$ contributed to the experimental formation of AD-like amyloid fibrils *in vivo*.

Author	Aβ Sequence	Target brain region	Major Conclusion
(Abe et al., 1994)	1-28, 25-35, 1-40	Medial Septum	Decreased hippocampal ACh release
(Clemens et al., 1992)	1-40	Hippocampus, striatum	Lack of Aβ toxicity
(Delobette et al., 1997)	1-28, 25-35, 1-42	i.c.v.	Conformation dependent memory impairment
(Emre et al., 1992)	1-40	Cortex	Acute neurotoxicity
(Frautschy et al., 1991)	Human Aβ extract	Hippocampus, cortex	Neuronal loss, tau phosphorylation
(Games et al., 1992)	25-35, 1-38, 1-40	Hippocampus, cortex	Lack of AD-related pathology
(Giovannelli et al., 1995)	25-35, 1-40	MBN	Conformation dependent toxicity
(Harkany et al., 1995)	1-42	MBN	Cholinergic toxicity
(Itoh et al., 1996)	1-40	Cholinergic systems	Reduced neurotransmitter release
(Nakamura et al., 2001)	1-42	i.c.v.	Reduced HACU after 25 days, reduced HACU and ChAT activity after 80 days
(Nitta et al., 1994) (Nitta et al., 1997)	1-40	i.c.v.	Decreased ChAT activity, learning deficit
(Stein-Behrens et al., 1992)	25-35	Hippocampus	Lack of hippocampal neuronal loss and Aβ neurotoxicity
(Winkler et al., 1994)	1-42	i.c.v., hippocampus	Lack of Aβ neurotoxicity

Table 3.1. Major findings from *in vivo* studies directed towards the identification of Aβ neurotoxicity in rat brain. A variety of shorter Aβ fragments have been used in these studies based on findings that the neurotoxic effect of Aβ may be mediated by short amino-acid sequences within the full Aβ sequence (Abe et al., 1994; Flood et al., 1994; Yankner et al., 1989) Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; ChAT, choline acetyltransferase; i.c.v., intracerebrolventricular; HACU, high affinity choline uptake; MBN, magnocellular basal forebrain neurons.

The finding that cholinergic neurons may be selectively vulnerable to the neurodegenerative processes underlying AD and that the loss of cholinergic neurons seen in AD is strongly correlated with the clinical symptoms (Flynn et al., 1986; Perry et al., 1977; for review see Bartus et al., 1982) has generated a great deal of interest into the relationship between Aβ deposition and cholinergic nerve damage. ChAT has been widely used as a cholinergic marker in studies that have examined the *in vivo* neurotoxic of effect of Aβ specifically in relation to cholinergic neurons. The results from these studies have been somewhat inconclusive. While some authors have reported a mild but significant decline in ChAT activity following injections of Aβ peptides into rat brain (Giovannelli et al., 1995; Harkany et al., 1995; Nitta et al., 1994; Pepeu et al., 1996; Yamada et al., 1998) others have reported no change (Abe et al., 1994; Winkler et al., 1994). Clearly this is an area that warrants further investigation. It has been the intention of the work presented here to contribute to that investigation by examining how chronic (14 day) *in vivo* treatment with a physiologically relevant dose of the major secreted form of the Aβ peptide ($A\beta_{(1\text{-}40)}$) affects cholinergic neuron viability as assessed by ChAT activity and AChE levels. A dose of 300 pmol/day for the Aβ infusions was chosen based on ELISA measurements showing that the physiological levels of soluble Aβ in culture medium and human cerebrospinal fluid (CSF) are in the high picomolar to low nanomolar range (Seubert et al., 1992; Vigo-Pelfrey et al., 1993).

A wide variety of mechanisms have been suggested to underlie the apparent neurotoxic effects of Aβ peptides including: activation of microglial cells (Meda et al., 1995); stimulation of tyrosine phosphorylation of focal adhesion kinase (Zhang et al., 1994a); inhibition of ubiquitin-dependent protein degradation (Gregori et al., 1995); induction of inflammatory responses (Deb et al., 1996; London et al., 1996); and generation of reactive oxygen species (Behl et al., 1994; Goodman et al., 1994b; Pappolla et al., 1997). Both apoptotic (Cotman et al., 1995) and necrotic (Behl et al., 1994) modes of

cell death have been proposed. A useful review of this area has recently been published (Vickers et al., 2000). There have also been a number of studies which report that Aβ may cause neurotoxicity by increasing $[Ca^{2+}]_i$ levels leading to pathological events associated with Ca^{2+} overload (Arispe et al., 1993; Fukuyama et al., 1994; Ito et al., 1994; Mattson et al., 1993b; Mattson et al., 1992). Alternatively, Aβ mediated increase in neuronal $[Ca^{2+}]_i$ on its own may not cause neurotoxicity, but may render neurons vulnerable to pathological Ca^{2+} overload when coupled with other mechanisms that raise $[Ca^{2+}]_i$. A number of studies have reported that levels of Aβ peptides below the concentrations required for direct neurotoxicity *in vitro* cause cultured neurons to become hypersensitive to NMDA mediated excitotoxicity (Gray et al., 1995; Koh et al., 1990; Patel, 1995a). Furthermore, NMDA receptor antagonists have been shown to protect against Aβ mediated neurotoxicity (O'Mahony et al., 1998). Although it has been reported that NMDAR levels are reduced in AD brains (Krystal et al., 1999; Sze et al., 2001), there is little information in the available literature concerning a direct interaction between Aβ peptides and NMDARs. One of the aims of the work presented here has been to investigate the potential for such an interaction by examining how the expression of NMDAR subunit mRNA is affected by chronic *in vivo* treatment with the $A\beta_{(1-40)}$ peptide.

Emerging evidence suggests that, in addition to any neurotoxic activity, Aβ peptides may act directly as neuromodulators of cholinergic neuron activity through mechanisms such as impairment of ACh release (Abe et al., 1994; Itoh et al., 1996; Kar et al., 1998) and decreased high affinity choline uptake (HACU) into cholinergic terminals (Kar et al., 1998). See Auld et al., (1998) for review. The mechanisms underlying these effects are not understood. It has also been reported that Aβ peptides competitively bind to (Wang et al., 2000a; Wang et al., 2000b) and inhibit the function of (Liu et al., 2001; Pettit et al., 2001) the α_7 nAChR subtype. The finding that in addition to the accumulation of Aβ in AD, there is a significant loss of nAChR sites (Flynn et al., 1986; Guan et al., 2000;

Nordberg et al., 1990; Nordberg et al., 1986; Sugaya et al., 1990; Warpman et al., 1995) and that this loss is correlated with cognitive deficits (Whitehouse et al., 1995) has prompted a number of investigations into a potential interaction between Aβ peptides and nAChRs. Results from a co-localisation study have shown that neurons expressing α_4 and α_7 nAChR mRNA were located in the vicinity of, or even inside Aβ immunoreactive plaques (Wevers et al., 1999). However, It is not clear whether Aβ peptides may contribute directly to nAChR loss in AD. This is an area that has been investigated as part of the work presented in this chapter.

Following reports that smoking is positively correlated with a delayed onset of AD (van Duijn et al., 1991), a number of laboratories have investigated the possibility that nicotine may possess neuroprotective properties in relation to Aβ mediated neurotoxicity (for reviews see Shimohama et al., 2001; Zamani et al., 2001). Subsequently, it has been reported that Aβ neurotoxicity can be partially, or even completely, prevented *in vitro* by nAChR activation in neuronal cell cultures (Kihara et al., 1997; Kihara et al., 1998; Zamani et al., 1997). The neuroprotective effects of nicotinic ligands have been attributed to both the $\alpha_4\beta_2$ (Kihara et al., 1998) and α_7 (Meyer et al., 1998b) nAChR subtypes in studies using selective ligands for these receptors. It is certainly notable that the existing data concerning the neuroprotective effect of nicotine in relation to Aβ toxicity has been generated *in vitro*. It has not been demonstrated conclusively that nAChR activation can prevent Aβ neurotoxicity *in vivo*. In addition to examining the apparent neurotoxic affect of Aβ, the work presented here has investigated whether chronic nicotine treatment (21 days) *in vivo* may modulate this effect, or indeed any other changes in various neurochemical markers mediated by Aβ treatment *in vivo*. The doses of chronic nicotine treatment of 0.75 and 1.25 mg/kg/day were chosen based on their relevance to concentrations of nicotine required for receptor activation and plasma concentrations typically seen in smokers. An indication of the plasma and brain concentrations achieved

with these doses is shown in table 3.2. Nicotine accumulates in the brain and consequently, for a given dose of nicotine, the concentration in the brain is generally about 3 times higher than plasma levels (Benowitz et al., 1990). The concentration of nicotine found in the plasma of smokers is highly variable but is usually in the order of 100 – 500 nM (Henningfield et al., 1983). The K_i values for (–)-nicotine at the major $\alpha_4\beta_2$ and α_7 nAChR subtypes in rat brain/human cell line are 1 nM and 5/1.6 μM respectively (Anderson et al., 1994; Gopalakrishnan et al., 1995a; Gopalakrishnan et al., 1995b). Reported EC_{50} values for (–)-nicotine in relation to its ability to stimulate neurotransmitter release in the brain vary from nanomolar concentrations for dopamine and glutamate release (Clarke et al., 1996; Grady et al., 1992; McGehee et al., 1995a; Whiteaker et al., 1995) up to micromolar concentrations for noradrenaline, acetylcholine, γ-aminobutyric acid and serotonin release (Clarke et al., 1996; Loiacono et al., 1990; Lu et al., 1998b; Wilkie et al., 1996; Yu et al., 1994).

Infusion dose (mg/kg/day)	Plasma concentration (nM)	Brain concentration (~ nM)
0.6	55	160
1.2	154	412
2.4	265	739

Table 3.2. Plasma and brain nicotine concentrations in the rat following continuous subcutaneous infusion (Rowell et al., 1997).

There has been surprisingly little data published concerning how Aβ peptides may affect the expression or processing of APP and therefore further Aβ generation. It has been reported that there is a significant reduction in APP levels (Davidsson et al., 2001) as well as the expression of PS1 and PS2 mRNA (Barton et al., 1996; Isoe-Wada et al., 1999; McMillan et al., 2000; Takami et al., 1997) in the brains of AD patients relative to age-matched controls. It is not known whether Aβ peptides contribute to this effect. Indeed,

there is little information concerning the possibility that Aβ peptides may directly affect the function or expression of proteins that are important for the processing of APP, such as BACE, TACE and the presenilins. This is another area that has been investigated as part of the present study. Specifically, the effect of chronic Aβ treatment *in vivo* on the expression of APP695, BACE, TACE, PS1 and PS2 was examined. Furthermore, the effect of chronic nicotine treatment on the mRNA expression of these proteins was also examined based on preliminary evidence detailed in a study by Kim et al., (1997) showing that acute nicotine treatment can stimulate the processing of APP.

While there has been a considerable amount of data published describing how many neurochemical markers are changed in AD brains relative to age-matched controls, relatively little is known about the potential for Aβ peptides to directly affect these changes. Furthermore, much of the work that has examined the biological effects of Aβ peptides has been done *in vitro*. The central aim of the work presented in this chapter has been to assess the effects of continuous i.c.v. Aβ infusion, chronic nicotine exposure and a combination of these treatments *in vivo* on a number of neurochemical markers relevant to AD, in specific anatomical areas selectively affected in AD.

3.2 METHODS

3.2.1 TREATMENT OF ANIMALS AND TISSUE PREPARATION

Sprague-Dawley rats (250 – 300 g) of either sex were arranged into 6 groups. Groups included: Sham-operated, β-amyloid$_{(1-40)}$ infused, 0.75 mg/kg/day nicotine, β-amyloid$_{(1-40)}$ infused + 0.75 mg/kg/day nicotine, 1.25 mg/kg/day nicotine and β-amyloid$_{(1-40)}$ infused + 1.25 mg/kg/day nicotine. Each group consisted of 4 animals.

Vehicle and β-amyloid$_{(1-40)}$ infused animals were anaesthetised with pentobarbitone (60 mg/kg i.p.) and constrained to a tilted skull position (-0.3 mm) using a Kopf stereotaxic apparatus. A continuous intracerebroventricular (i.c.v.), 14 day infusion of either β-amyloid$_{(1-40)}$ 300 pmol/day (RBI, USA) dissolved in a vehicle of 30% acetonitrile/0.1% trifluoroacetic acid (v/v) or vehicle alone was achieved using brain infusion kits (Alza corp., USA.) attached to modified miniosmotic pumps (pump rate of 1 μl/hr; Alza corp., USA). The osmotic minipumps were weighed before and after addition of the solution for i.c.v. infusion to ensure that they had been filled. After removing the plastic cap that covers the metal rod housed in the minipump a 4.5 cm length of polyethelene tubing was used to attach the minipumps to the infusion cannula. The minipumps were placed between the scapulae in a small subcutaneous cavity. A small hole was drilled into the skull and a capped infusion cannula was implanted into the left lateral ventricle at stereotaxic coordinates: 1.0 mm caudal to bregma, 1.5 mm left from the midline, 3.4 mm ventral from the cortical surface according to the atlas of Paxinos and Watson (1986). The infusion cannula was fixed in place by inserting a small stainless steel screw into the skull and then anchoring the cannula to the screw using dental cement.

All animals receiving chronic nicotine treatment were implanted subcutaneously with 7-day osmotic minipumps which were replaced with 14 day miniosmotic pumps on day 7 to give a total of 21 days treatment. Nicotine was dissolved in 10 mM PBS. For the groups that received both chronic nicotine treatment and i.c.v. infusions, the infusion kits were implanted on day 7 of chronic nicotine treatment. Therefore, $A\beta_{(1-40)}$ treated animals received chronic nicotine treatment 1 week prior to and for the duration the $A\beta_{(1-40)}$ infusions (Fig 3.0).

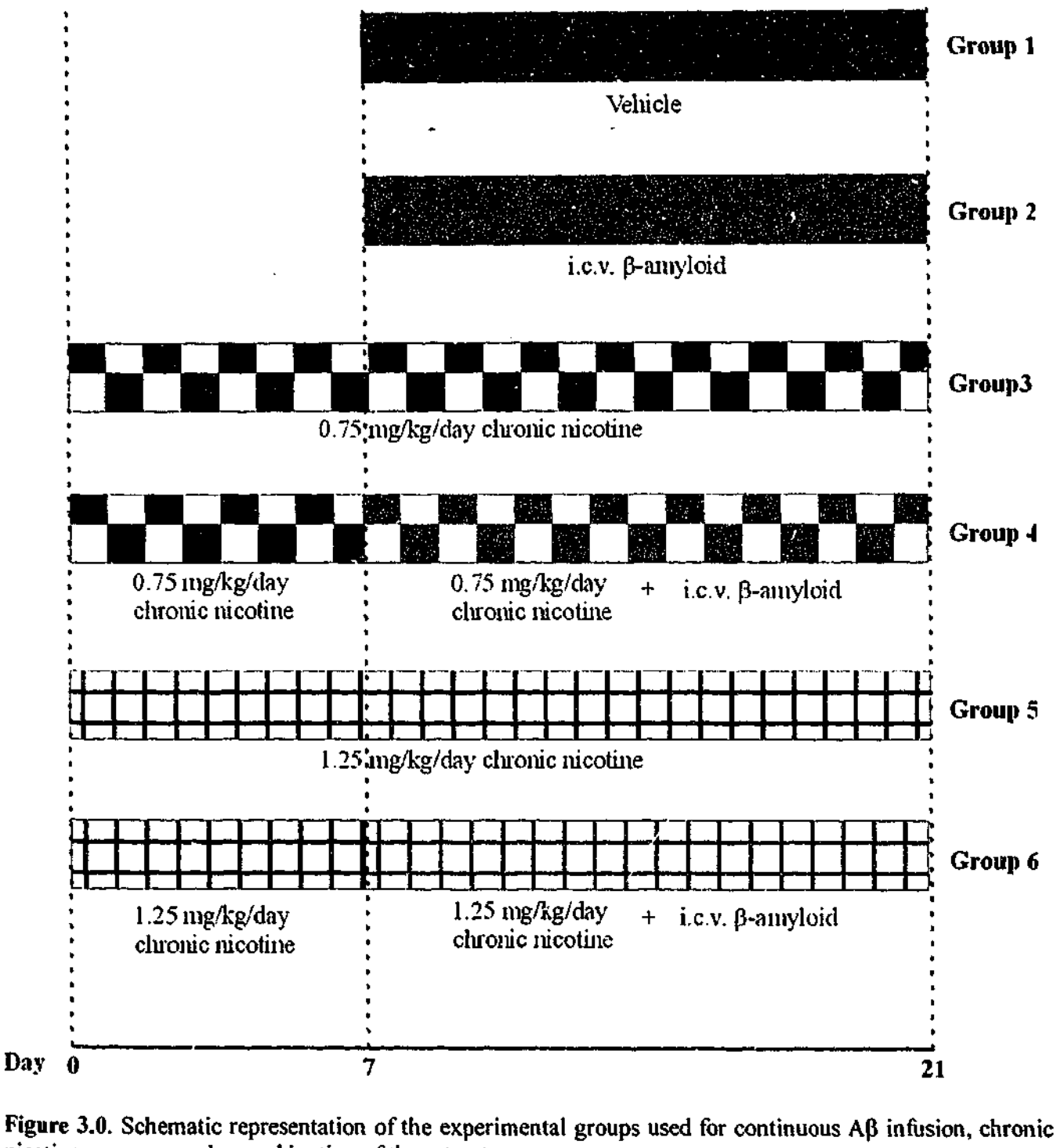


Figure 3.0. Schematic representation of the experimental groups used for continuous Aβ infusion, chronic nicotine exposure and a combination of these treatments.

Following treatment, animals were decapitated and the brains frozen over liquid nitrogen. For procedures requiring homogenate preparations, the hippocampus and frontal cortex was isolated prior to freezing. For histochemical and autoradiographic analysis, a Reichert Jung cryostat was used to cut a series of 14 μm sections. Consecutive coronal sections were taken at 3.6 mm caudal to bregma and thaw mounted on poly-L-lysine coated slides. All tissue was stored at -70°C until required.

3.2.2 AChE Histochemistry

AChE levels were quantified using a modification of the method previously described by Koelle et al., (1949). This method has been described in detail under Chapter 2, General Methodology, *2.4 AChE histochemistry.*

3.2.3 Radioenzymatic ChAT Quantification

ChAT activity was measured according to the method originally described by Fonnum, (1975). Hippocampus or frontal cortex was homogenised at 5% w/v in 10 mM EDTA (ph 7.4) with 0.5% v/v Triton X-100 using a glass homogeniser. The homogenates were centrifuged at 10 000g for 15 minutes and 2 μl of the supernatent was mixed with 5 μl of a substrate solution consisting of 0.25 mM [^{3}H]acetyl coenzyme A ([^{3}H]acetyl-CoA; 200 mCi/mmol; NEN™ Life Science Products Inc., USA), 8 mM choline, 20 mM EDTA, 300 mM NaCl and 0.1 mM eserine in 50 mM phosphate buffer (50 mM Na_2HPO_4/50 mM NaH_2PO_4; pH 7.4). The reaction proceeded for 15 min at 37°C after which time the mixture was washed with 5 ml of 10 mM PBS (pH 7.4) and transferred to scintillation

vials. This was followed by the addition of 2 ml of acetonitrile containing 10 mg of sodium tetraphenyl boron and 10 ml of toluene scintillation mixture (0.05% diphenyloxazole/0.02% 1,4-bis-(4-methyl-5-phenyloxazole-2-yl)benzene). The vials were shaken and left overnight to allow separation of the newly synthesised [^{3}H]ACh contained in the toluene phase and the [^{3}H]acetyl-CoA contained in the aqueous phase before counting in a Packard Tri-Carb liquid scintillation spectrophotometer with a counting efficiency of 30-50%. Differences in the amount of ChAT activity between the vehicle group and the treatment groups, as indicated by the amount of [^{3}H]ACh synthesised, were assessed statistically using one-way ANOVA with a Dunnett post test for multiple comparisons. Statistical significance was signified by P values of less then 0.5. Non-enzymatic activity, determined using boiled tissue, was subtracted from the total activities prior to statistical analysis.

3.2.4 [^{3}H]-HEMICHOLINIUM AUTORADIOGRAPHY

Measurement of the density of high affinity choline uptake sites was based on the methods previously described by Quirion, (1987). Slide mounted sections were incubated for 60 min at 4°C in 50 mM Tris-HCl (pH 7.4) containing 300 mM NaCl and 15 nM Methyl-[^{3}H]hemicholiniun-3 diacetate salt ([^{3}H]HC-3; specific activity > 120 Ci/mmol; NEN™ Life Science Products Inc., USA). To determine non-specific binding, 10 μM non-radioactive HC-3 was included in adjacent sections. Following incubation, slides were taken through 8 x 2 min rinses in ice-cold incubation buffer, rinsed in dH_20 and dried immediately under a stream of air. Slides were apposed to tritium-sensitive film (Hypermax Hyperfilm, Amersham, UK) for 3 weeks. A set of tritium scales (Amersham, USA) were also apposed to film for the same length of time. Films were processed at room

temperature in light-safe conditions as previously described: (5 min in a solution of Phenisol X-ray film developer (Ilford, Australia), rinsed in a mixture of water and stopbath (Ilford, Australia) for 1 min and fixed in hyperfilm rapid-fixer (Ilford, Australia) for 10 min). The resulting autoradiographic images were digitally captured and the density of high affinity choline uptake sites quantified in terms of nCi/mm^2 using Scion imaging software calibrated against the standard tritium scales. See Chapter 2, General Methodology, *2.5 Quantitation* for a more detailed account of image analysis.

3.2.5 In Situ Hybridisation Histochemistry

The protocol for in situ hybridisation is based on the methods described by Wisden et al., (1994) and adapted by Loiacono et al., (1999), and has been described in detail under Chapter 2, General Methodology, *2.1 In Situ Hybridisation Histochemistry.*

3.2.6 [^{3}H]Epibatidine Autoradiography

The protocol for [^{3}H]epibatidine autoradiography was based on that described by Perry et al., (1995b) and has been described in detail under Chapter 2, General Methodology, *2.2 [^{3}H]Epibatidine Autoradiography.*

3.2.7 [^{125}I]α-Bungarotoxin Autoradiography

The protocol for [^{125}I]α-BGT was based on that used by Whiteaker et al., (1999) and has been described in detail under Chapter 2, General Methodology, *2.3 [^{125}I]α-Bungarotoxin Autoradiography.*

3.2.8 *QUANTITATION*

Images generated through the ISSH, histochemical and binding protocols were digitally captured and selected anatomical regions were quantified using Scion imaging software. For mRNA images, [^{125}I]α-BGT autoradiographs and AChE histochemical stains the system was calibrated against a set of graded greyscales (Kodak), allowing for quantitation in terms of relative optical density (ROD). For [^{3}H]epibatidine and [^{3}H]HC-3 autoradiographs, the system was calibrated against a set of standard tritium scales, allowing for quantitation in terms of nCi/mm^2. The mean ROD or nCi/mm^2 measurements for each treatment group were compared statistically using a one-way ANOVA with a Tuckey test for multiple comparisons. Statistical significance was signified by P values of less then 0.5. The value in all treatment groups represents the mean of measurements taken from 4 animals. The symbol, '*', has been used to denote statistically significant differences between a particular treatment group and the vehicle treated group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Where there were statistically significant differences between the vehicle group and multiple treatment groups, any significant differences in the means between the various treatment groups have also been reported. The symbol, '†', has been used to denote statistically significant differences between each treatment group (†, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$). Issues relating to quantitation have been described in greater detail in Chapter 2, General Methodology, *2.5 Quantitation.*

3.3 RESULTS

3.3.1 AChE LEVELS

AChE levels were quantified through densitometric analysis in the Ca1, Ca2 and Ca3 regions of the hippocampus, the granular and molecular layers of the dentate gyrus, the hippocampal fissure and cortical layers 1-3 and 4-6 as defined in the atlas of Paxinos and Watson (1986). The anatomical distribution of AChE in telencephic regions of the rat brain is shown in a representative coronal section (fig. 3.1).

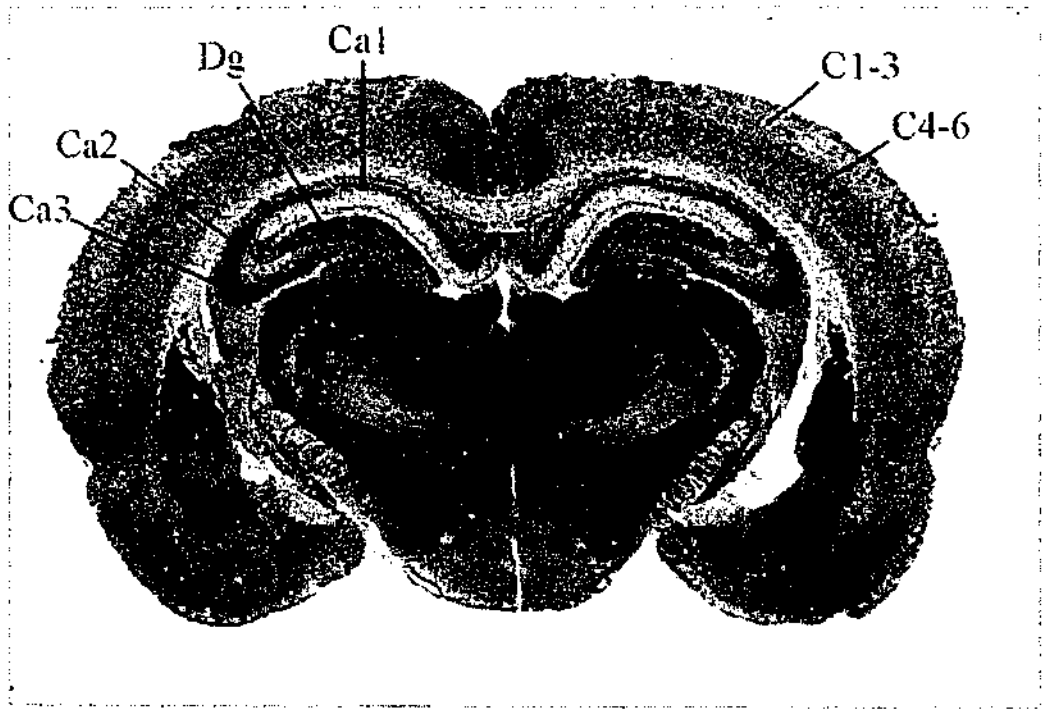


Figure 3.1. AChE histochemistry in a representative coronal section approximately 3.6 mm caudal to bregma. Abbreviations: Ca1-3, fields of the hippocampus; c1-3, cortical layers 1-3; c4-6, cortical layers 4-6; Dg, dentate gyrus; HiF, hippocampal fissure; MolDG, dentate gyrus molecular layer. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

No significant change in AChE levels was seen relative to vehicle treated rats as a result of continuous $A\beta_{(1-40)}$ infusion, chronic nicotine treatment or a combination of these treatments (one way ANOVA with a Tuckey post-test, $P > 0.05$; fig. 3.2).

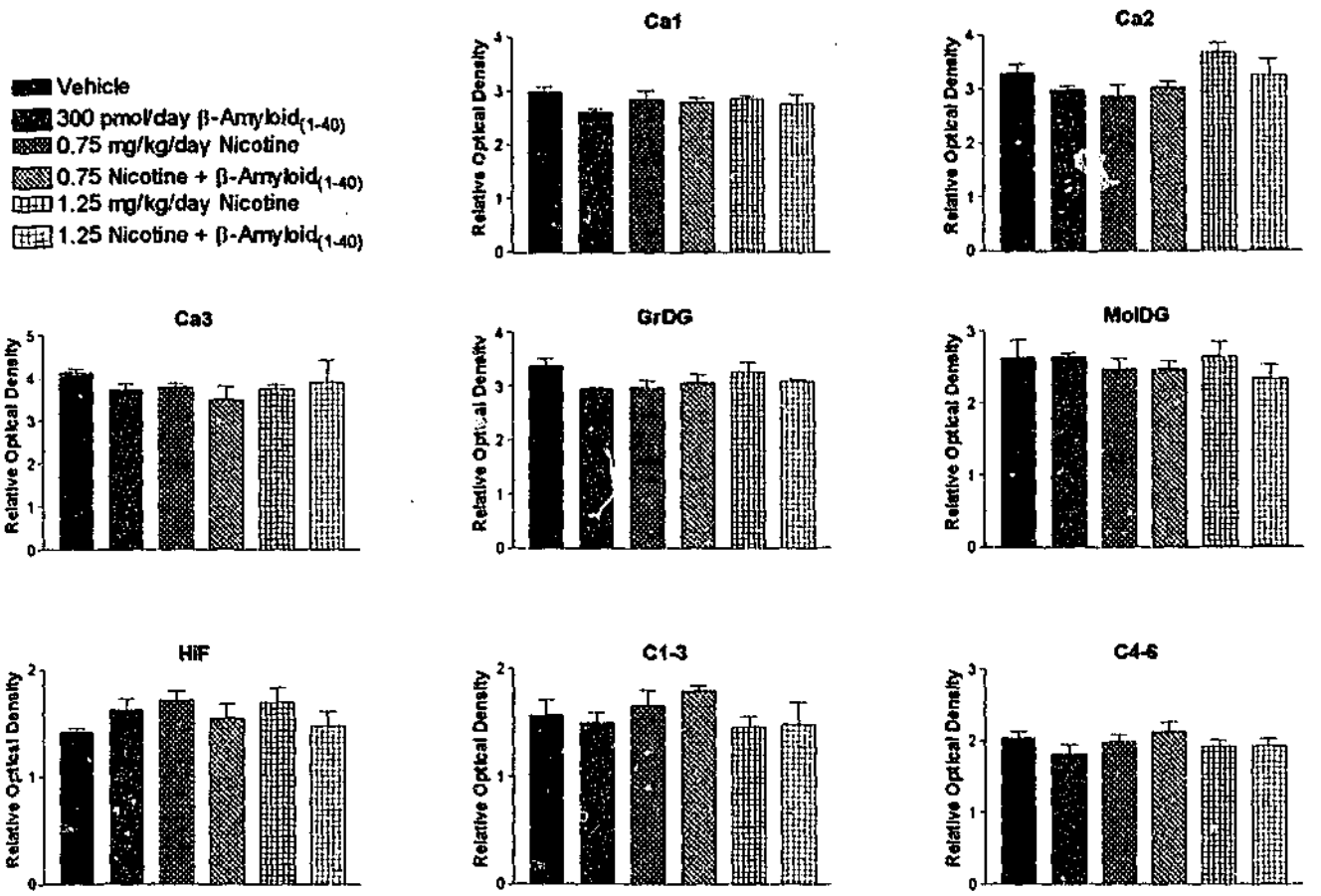


Figure 3.2. AChE levels, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus; c1-3, cortical layers 1-3; c4-6, cortical layers 4-6; HiF, hippocampal fissure; MolDG, dentate gyrus molecular layer. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post-test. Differences in the means were not statistically significant in all cases ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

3.3.2 ChAT Activity

ChAT activity was assessed in homogenates of hippocampus or cortex from the left hemisphere. None of the treatment groups showed significantly different ChAT activity relative to vehicle in either the hippocampus or cortex (one-way ANOVA with a Tuckey post-test, $P > 0.05$; fig. 3.3). Only the higher 1.25 mg/kg/day dose of nicotine was used in

combination with $A\beta_{(1-40)}$ treatment. Non-enzymatic activity, determined using boiled tissue, was less than 10% of total radioactivity (data not shown).

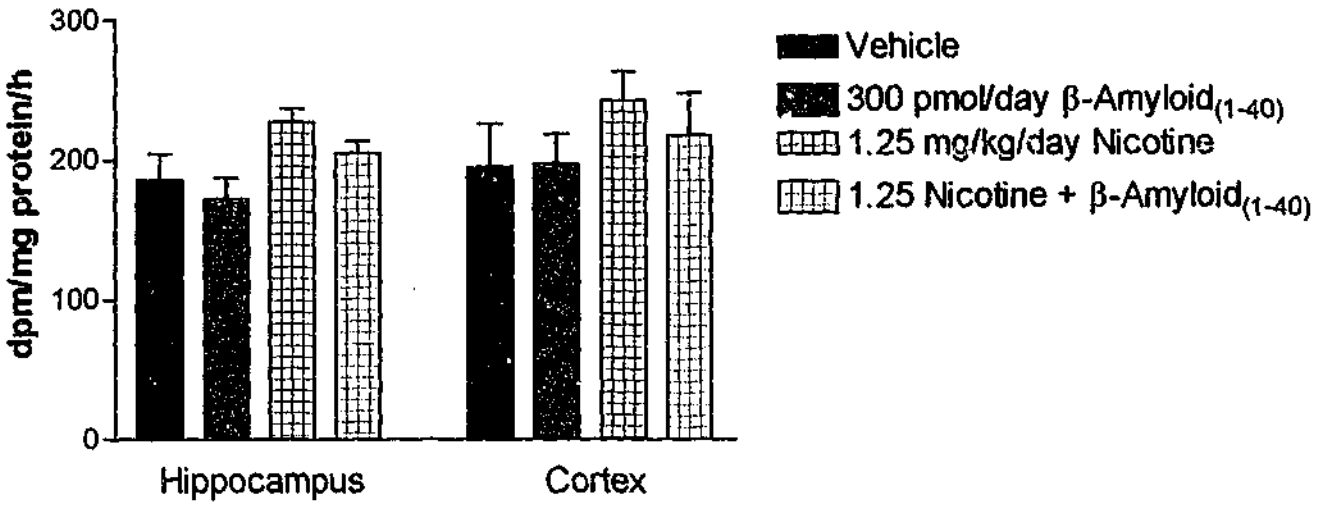


Figure 3.3. ChAT activity in hippocampal and cortical membrane fragments following treatment with chronic nicotine (1.25 mg/kg/day, 21 days), β-amyloid$_{(1-40)}$ (i.c.v., 14 days) or a combination of these treatments. The mean ChAT activity for the vehicle group was compared to the mean ChAT activity for each treatment group using one-way ANOVA with a Tuckey post test. Differences in the means were not statistically significant in all cases ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

3.3.3 [^{3}H]Hemicholinium Autoradiography

The density of high affinity choline uptake sites in various telencephic regions in the rat brain was measured by [^{3}H]HC-3 autoradiography. Representative autoradiographs of [^{3}H]HC-3 binding from the various treatment groups are shown in figure 3.4. Non-specific binding typically accounted for approximately 40% of the total signal in the regions quantified and was subtracted from total binding during quantitation.

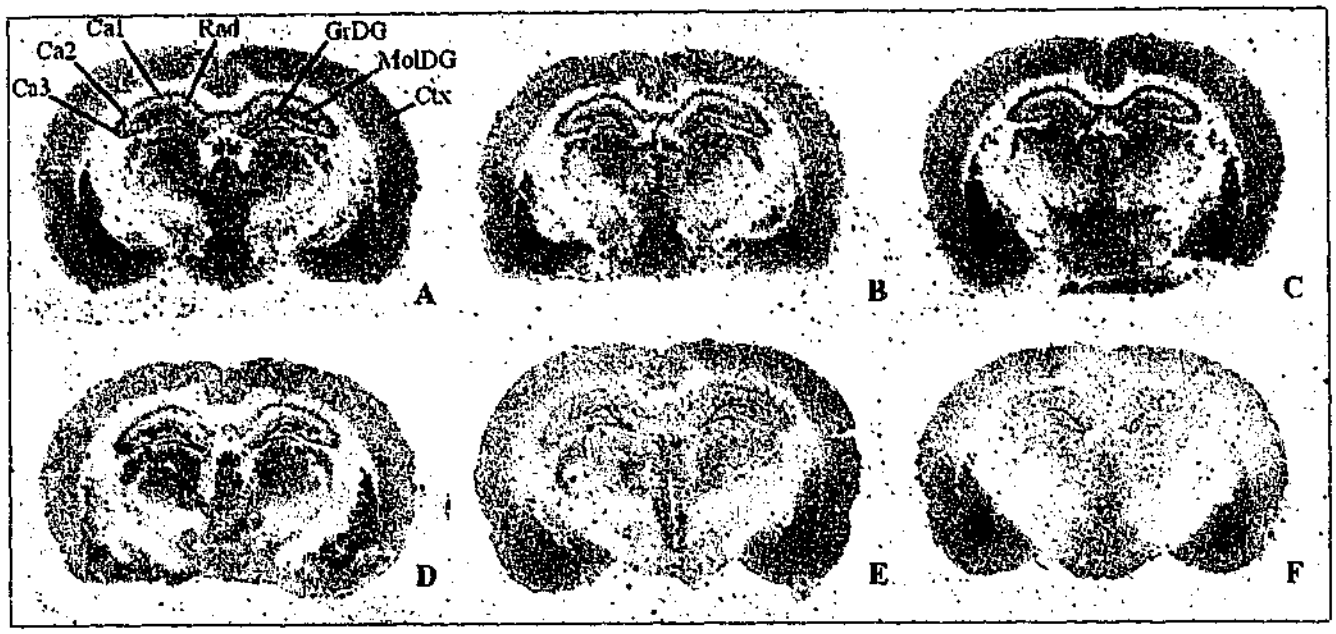


Figure 3.4 Total [^{3}H]HC-3 binding in representative coronal sections 3.6 mm caudal to bregma from vehicle (A), 300 pmol/day β-amyloid$_{(1-40)}$ (B), 0.75 mg/kg/day nicotine (C), 300 pmol/day β-amyloid$_{(1-40)}$ + 0.75 mg/kg/day nicotine (D), 1.25 mg/kg/day chronic nicotine (E) and 300 pmol/day β-amyloid$_{(1-40)}$ + 1.25 mg/kg/day nicotine treated animals. Abbreviations: Ca1-3, fields of the hippocampus; Ctx, frontal cortex; GrDG, dentate gyrus granular layer; MolDG, dentate gyrus molecular layer; Rad, stratum radiatum hippocampus.

Continuous i.c.v. infusion of A$\beta_{(1-40)}$ resulted in a modest reduction of approximately 13 – 20% in the various hippocampal and cortical regions quantified (fig. 3.5). This result was statistically significant in the Ca1, Ca2 and Ca3 fields of the hippocampus. Chronic nicotine treatment alone had no significant effect at a dose of 0.75 mg/kg/day but caused significant reductions in [^{3}H]HC-3 binding of 10 – 28% at the higher dose of 1.25 mg/kg/day in the Ca1, Ca2 and Ca3 fields of the hippocampus as well as the cortex. Animals which received chronic nicotine treatment at a dose of 0.75 mg/kg/day in addition to A$\beta_{(1-40)}$ showed a decrease in [^{3}H]HC-3 binding of approximately 11 – 27% which was statistically significant in the Ca1 and Ca2 regions of the hippocampus as well as the granular layer of the dentate gyrus and the cortex. While [^{3}H]HC-3 binding in this group was not significantly different to the A$\beta_{(1-40)}$ alone group, it is notable that a greater number of areas were significantly affected. In the A$\beta_{(1-40)}$ alone group, [^{3}H]HC-3 binding in the cortex, Ca1 field of the hippocampus and granular layer of the dentate gyrus was unaffected relative to control levels, however in the A$\beta_{(1-40)}$ + 0.75 nicotine group

there was a significant reduction in [^{3}H]HC-3 binding in these areas. Rats co-treated with both $A\beta_{(1-40)}$ and 1.25 mg/kg/day chronic nicotine exhibited a loss in [^{3}H]HC-3 binding that was significantly greater than was observed in rats treated with either $A\beta_{(1-40)}$ or 1.25 mg/kg/day chronic nicotine alone. Animals receiving $A\beta_{(1-40)}$ and 1.25 mg/kg/day chronic nicotine exhibited significant reductions in [^{3}H]HC-3 binding of 42 – 60% throughout the hippocampus and cortex. Notably, in areas not significantly affected by $A\beta_{(1-40)}$ or 1.25 mg/kg/day alone, such as the molecular layer of the dentate gyrus and the stratum radiatum area of the hippocampus, there was a significant reduction in [^{3}H]HC-3 binding as a result of $A\beta_{(1-40)}$ and 1.25 mg/kg/day co-treatment.

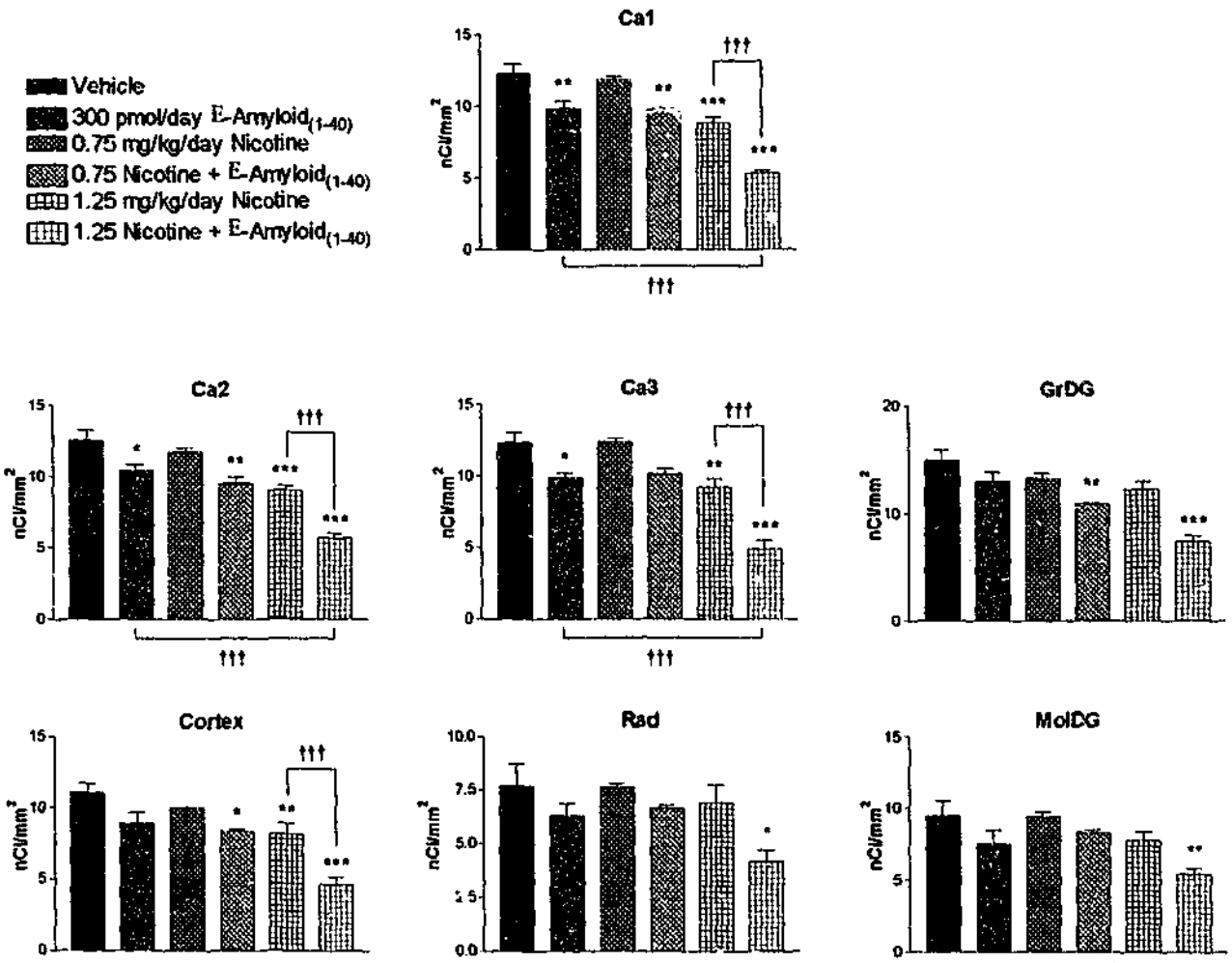


Figure 3.5. Specific [^{3}H]HC-3 binding in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus; GrDG, dentate gyrus granular layer; MolDG, dentate gyrus molecular layer; Rad, stratum radiatum hippocampus. The mean nCi/mm^2 for the vehicle group was compared to the mean nCi/mm^2 for each treatment group using one-way ANOVA with a Tuckey post-test (*, $P < 0.05$; **, $P < 0.01$; ***, 0.001). The same test was also used to compare the reduction in [^{3}H]HC-3 binding associated with the $A\beta_{(1-40)}$ + 1.25 mg/kd/day chronic nicotine group with that associated with both the $A\beta_{(1-40)}$ and 1.25 mg/kg/day chronic nicotine alone groups (†††, $P < 0.001$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

3.3.4 nAChR SUBUNIT mRNA EXPRESSION

nAChR subunit mRNA expression was examined by *in situ* hybridisation histochemistry in sections of brain from rats from each treatment group. Figure 3.6 shows the unique anatomical distribution of α_4, α_7 and β_2 nAChR subunit mRNA expression in various telencephic regions of the rat brain.

Figure 3.6. nAChR α_4, α_7, and β_2 mRNA subunit expression in representative coronal sections approximately 3.6 mm caudal to bregma. Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4, cortical layer 4; C5, cortical layer 5; C4-5, cortical layers 4-5; C6, cortical layer 6; Ctx, cortex; Dg, dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Infusions of $A\beta_{(1\text{-}40)}$, chronic nicotine or a combination of these treatments did not significantly affect the level of α_4 (fig. 3.7) or β_2 (fig. 3.8) mRNA expression (one-way ANOVA with a Tuckey post test, $P > 0.05$). Animals which received continuous $A\beta_{(1\text{-}40)}$ infusions in combination with chronic nicotine treatment at a dose of 1.25 mg/kg/day exhibited a small increase in α_7 expression of between 10 – 32% throughout the hippocampus and cortex (fig. 3.9) relative to vehicle treated animals. This effect was statistically significant in the Ca1 area of the hippocampus only. Treatment with chronic nicotine or β-amyloid$_{(1\text{-}40)}$ alone did not significantly effect α_7 nAChR subunit mRNA levels.

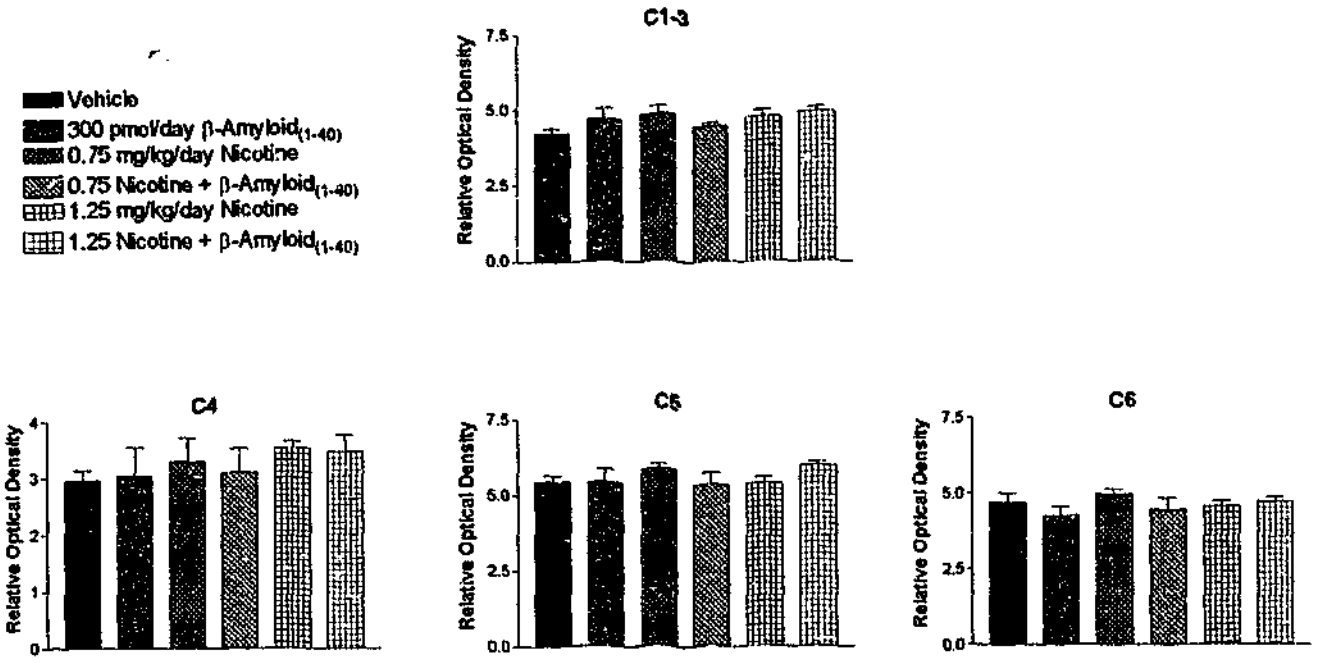


Figure 3.7. Levels of α_4 nAChR subunit mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4, cortical layer 4; C5, cortical layer 5; C5, cortical layer 5; C6, cortical layer 6. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post test. Differences in the means were not statistically significant in all cases ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

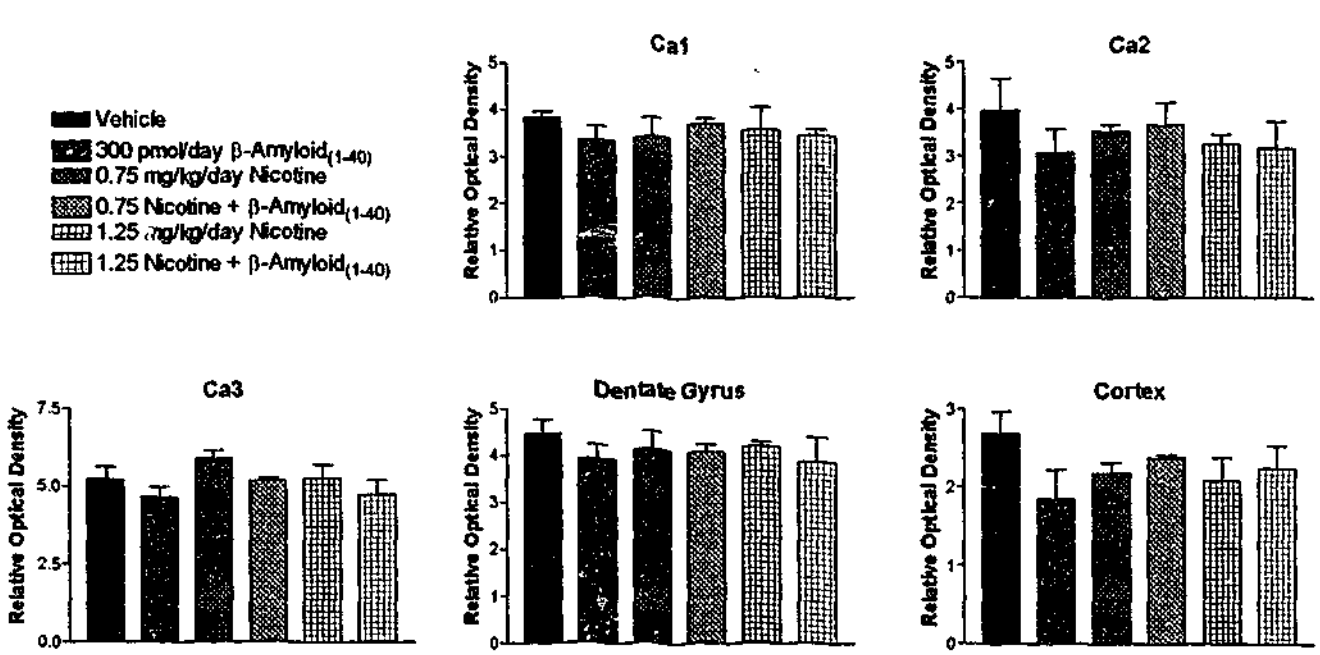


Figure 3.8. Levels of β_2 nAChR subunit mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus. The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post-test. Differences in the means were not statistically significant in all cases ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

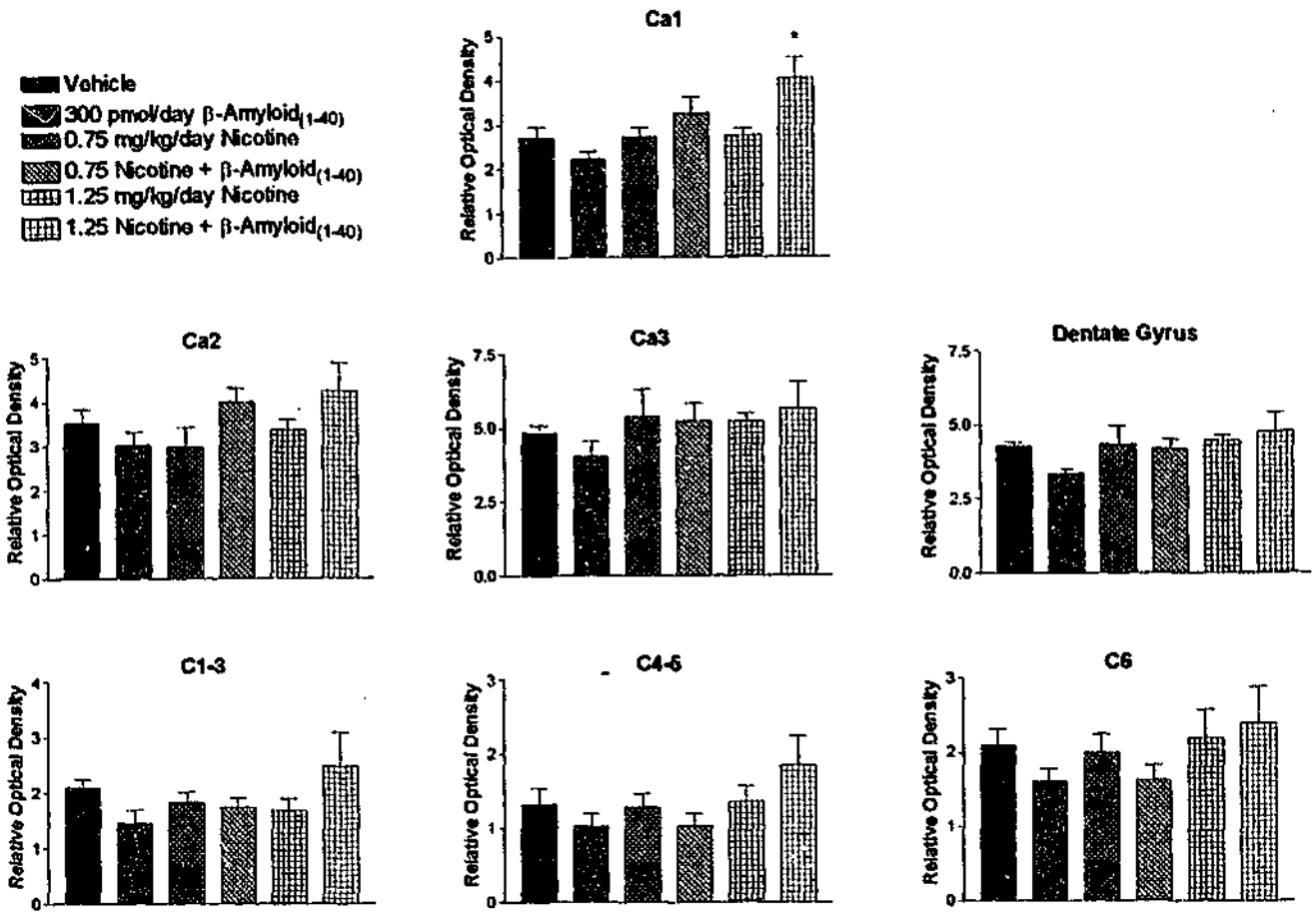


Figure 3.9. Levels of α_7 nAChR subunit mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4-5, cortical layer 4-5; C6, cortical layer 6. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post test (*, $P < 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

3.3.5 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY

Nicotinic receptor levels were assessed in rats from each treatment group by receptor autoradiography using [^{3}H]epibatidine. Epibatidine binds predominately to nAChRs of the $\alpha_4\beta_2$ subtype and also, to a lesser extent, to other subtypes such as the $\alpha_3\beta_2$ receptor, which are expressed at much lower levels. The regional distribution of [^{3}H]epibatidine binding in representative sections is shown in figure 3.12. Non-specific binding, as defined by 300 μM (–)-nicotine, was undetectable in all cases (data not shown).

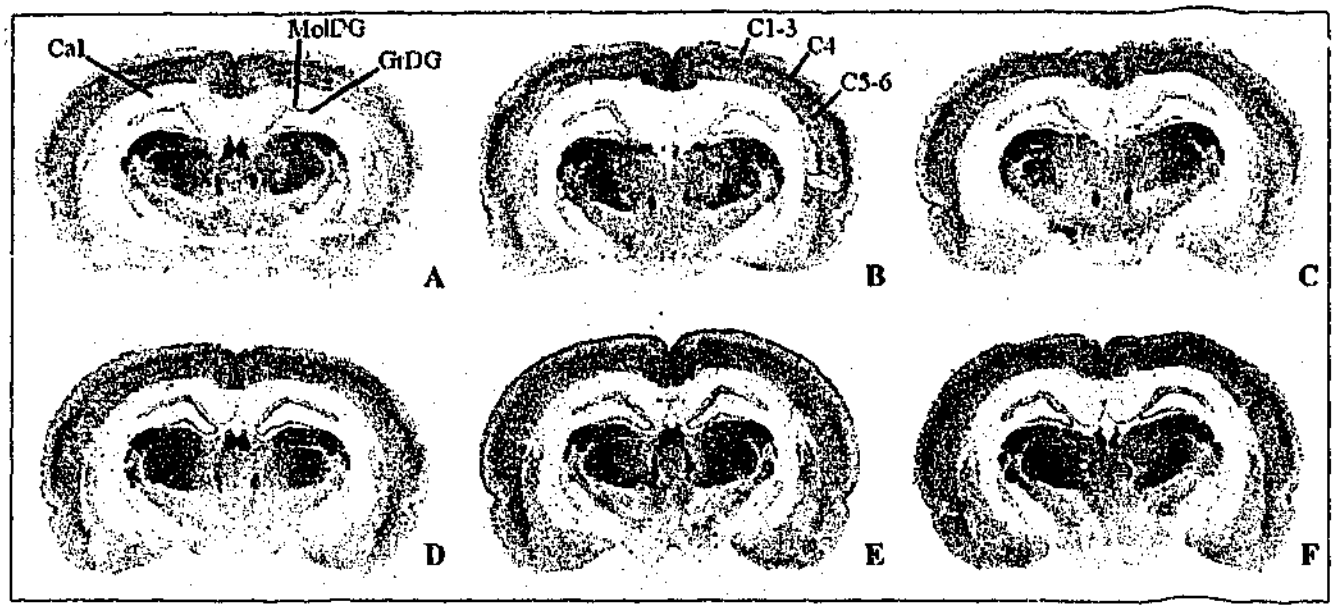


Figure 3.12. Total [^{3}H]Epibatidine binding in representative coronal sections approximately 3.6 mm caudal to bregma from vehicle (A), 300 pmol/day β-amyloid$_{(1-40)}$ (B), 0.75 mg/kg/day nicotine (C), 300 pmol/day β-amyloid$_{(1-40)}$ + 0.75 mg/kg/day nicotine (D), 1.25 mg/kg/day chronic nicotine (E) and 300 pmol/day β-amyloid$_{(1-40)}$ + 1.25 mg/kg/day nicotine treated animals. Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4, cortical layer 4; C5-6, cortical layers 5-6; GrDG, granular layer dentate gyrus; MolDG, molecular layer dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Although there was a small increase in [^{3}H]epibatidine binding in animals treated with i.c.v. infusions of $A\beta_{(1-40)}$ in all regions examined, this was not a statistically significant result. Both doses of chronic nicotine, however, caused a significant increase in [^{3}H]epibatidine binding throughout the hippocampus and cortex (fig. 3.13). There was no significant difference between the doses used in regard to this effect. Interestingly, in the molecular layer of the dentate gyrus, only the two groups which received both $A\beta_{(1-40)}$ and chronic nicotine treatment showed a significant increase in [^{3}H]epibatidine binding relative to treatment with vehicle only. The greatest increase in [^{3}H]epibatidine binding was consistently seen in the $A\beta_{(1-40)}$ + 1.25 mg/kg/day treatment group.

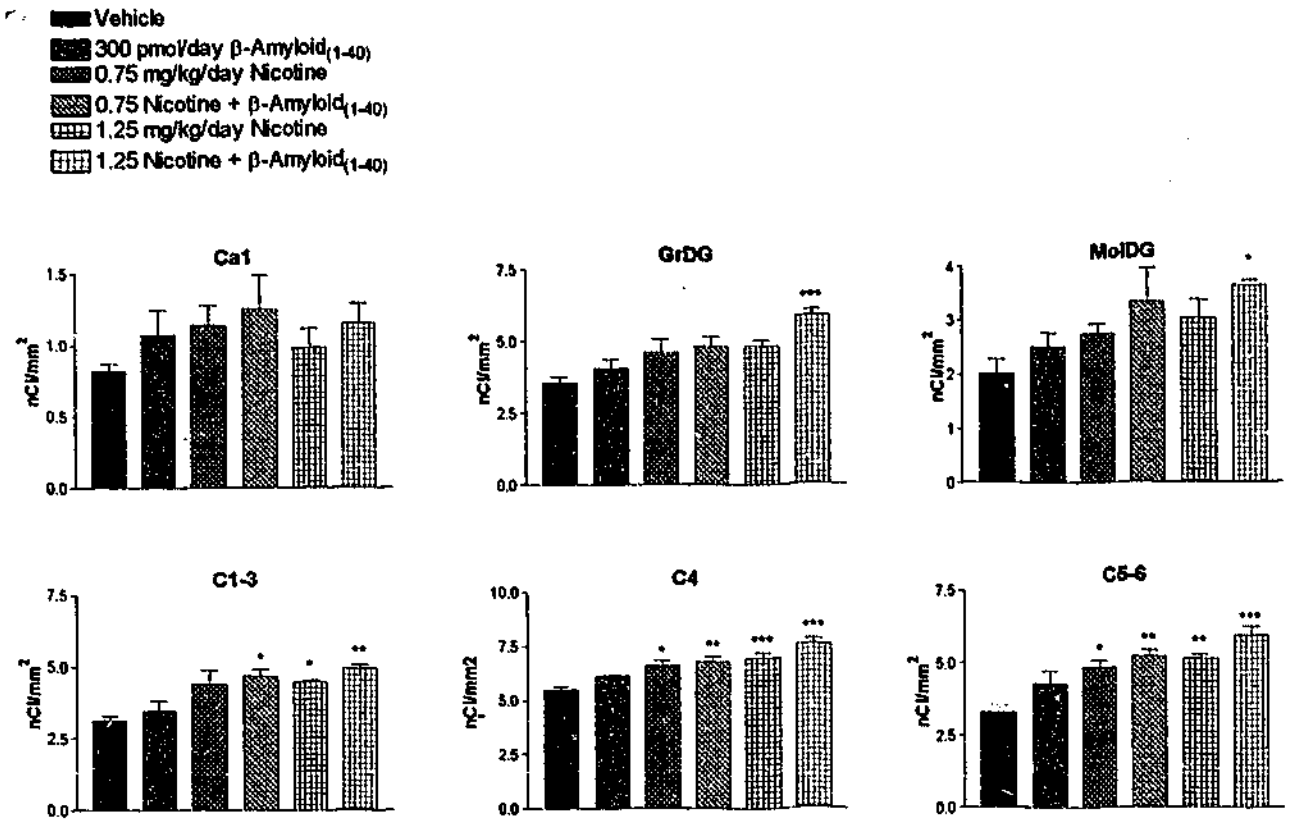


Figure 3.13. Levels of specific [^{3}H]epibatidine binding in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4, cortical layer 4; C5-6, cortical layers 5-6; GrDG, granular layer dentate gyrus; MolDG, moleculer layer dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean nCi/mm^2 for the vehicle group was compared to the mean nCi/mm^2 for each treatment group using one-way ANOVA with a Tuckey post-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The same test was also used to compare the increase in [^{3}H]epibatidine binding associated with the A$\beta_{(1-40)}$ + 0.75/1.25 chronic nicotine groups with that associated with the corresponding dose of chronic nicotine alone ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

3.3.6 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY

Levels of the α_7 nAChR subtype were assessed in the Ca1, Ca2, and Ca3 areas of the hippocampus, the hippocampal fissure, the stratum radiatum area of the hippocampus and cortical layers 1-5 and 6 as defined in the atlas of Paxinos and Watson (1986) in rats from each treatment group by receptor autoradiography using the α_7 nAChR selective ligand [^{125}I]α-BGT. The regional distribution of [^{125}I]α-BGT binding to α_7 nAChRs in a representative coronal section is shown in figure 3.10. Non-specific binding ranged from <10% in the dentate gyrus to approximately 40% in the stratum radiatum hippocampus and was subtracted from the total signal during quantitation.

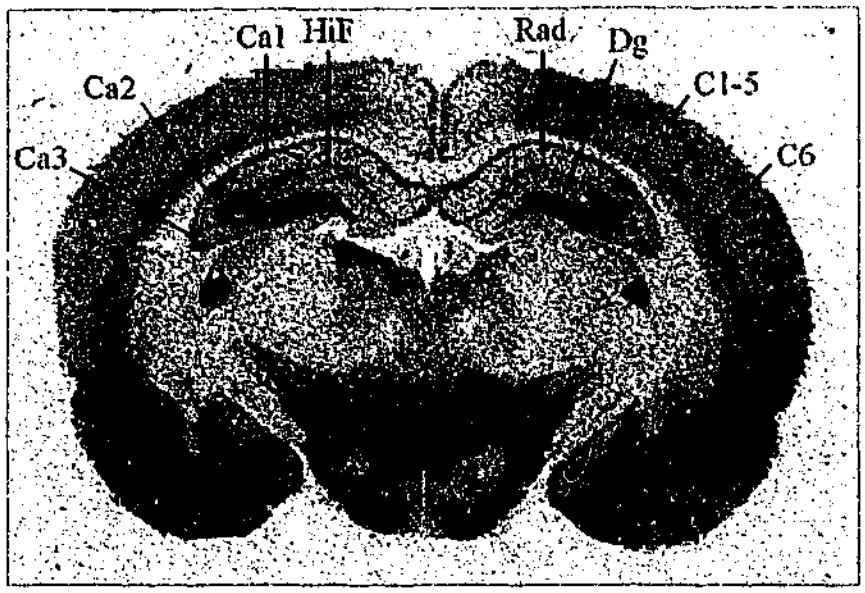


Figure 3.10. Total [^{125}I]α-BGT binding in a representative section approximately 3.6mm caudal to bregma. Abbreviations: Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

There was no significant change in the level of [^{125}I]α-BGT binding as a result of $A\beta_{(1\text{-}40)}$ infusions, chronic nicotine or a combination of these treatments relative to vehicle infused animals (one-way ANOVA with a Tuckey post-test, $P > 0.05$; fig. 3.11).

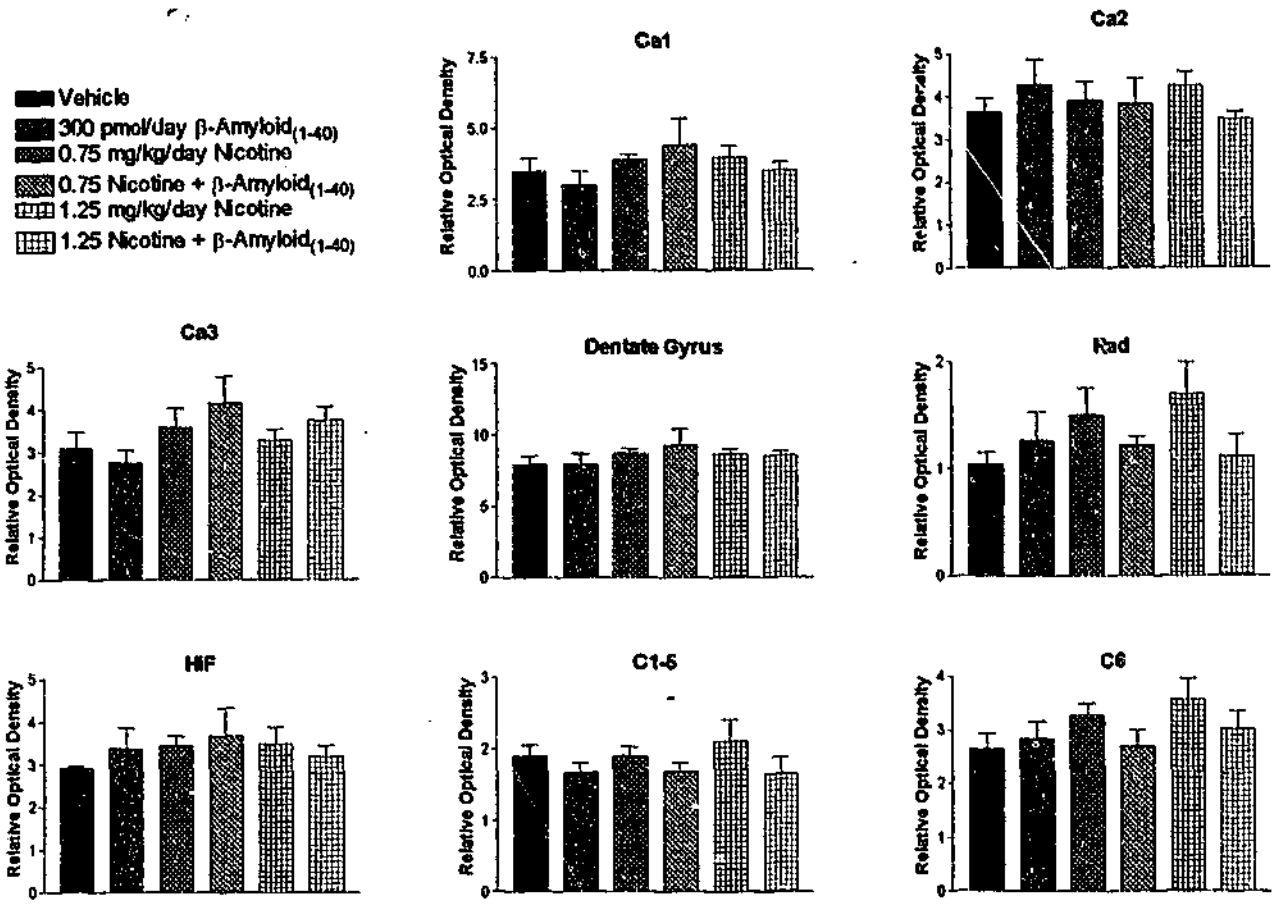


Figure 3.11. Levels of specific [^{125}I]α-BGT binding, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; HiF, hippocampal fissure; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post test. Differences in the means were not statistically significant in all cases ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

3.3.7 NMDA Receptor Subunit mRNA Expression

NMDAR subunit mRNA expression was examined by *in situ* hybridisation histochemistry in sections of brain from rats from each treatment group. Representative autoradiographs of NR1, NR2A and NR2B NMDAR subunit mRNA expression from each treatment group are shown in figures 3.14, 3.15 and 3.16 respectively.

NR1

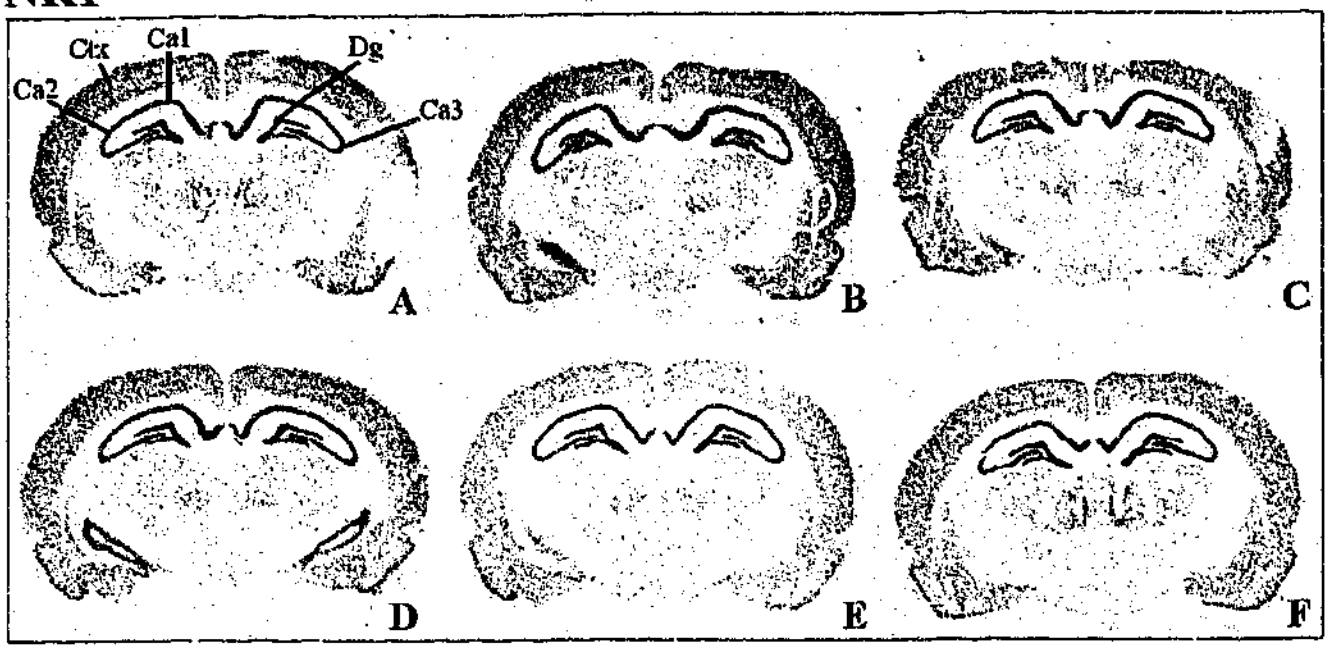


Figure 3.14. NR1 subunit mRNA expression in representative coronal sections 3.6 mm caudal to bregma from vehicle (A), 300 pmol/day β-amyloid$_{(1-40)}$ (B), 0.75 mg/kg/day nicotine (C), 300 pmol/day β-amyloid$_{(1-40)}$ + 0.75 mg/kg/day nicotine (D), 1.25 mg/kg/day chronic nicotine (E) and 300 pmol/day β-amyloid$_{(1-40)}$ + 1.25 mg/kg/day nicotine treated animals. Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus.

NR2A

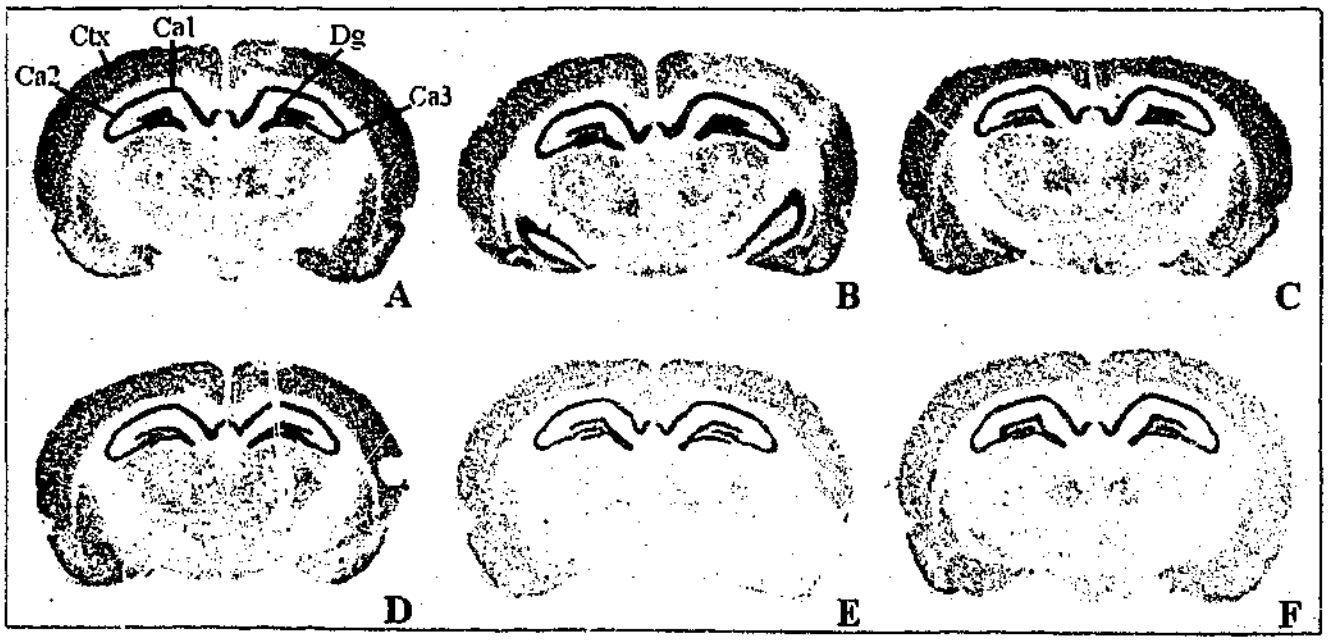


Figure 3.15. NR2A subunit mRNA expression in representative coronal sections 3.6 mm caudal to bregma from vehicle (A), 300 pmol/day β-amyloid$_{(1-40)}$ (B), 0.75 mg/kg/day nicotine (C), 300 pmol/day β-amyloid$_{(1-40)}$ + 0.75 mg/kg/day nicotine (D), 1.25 mg/kg/day chronic nicotine (E) and 300 pmol/day β-amyloid$_{(1-40)}$ + 1.25 mg/kg/day nicotine treated animals. Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus.

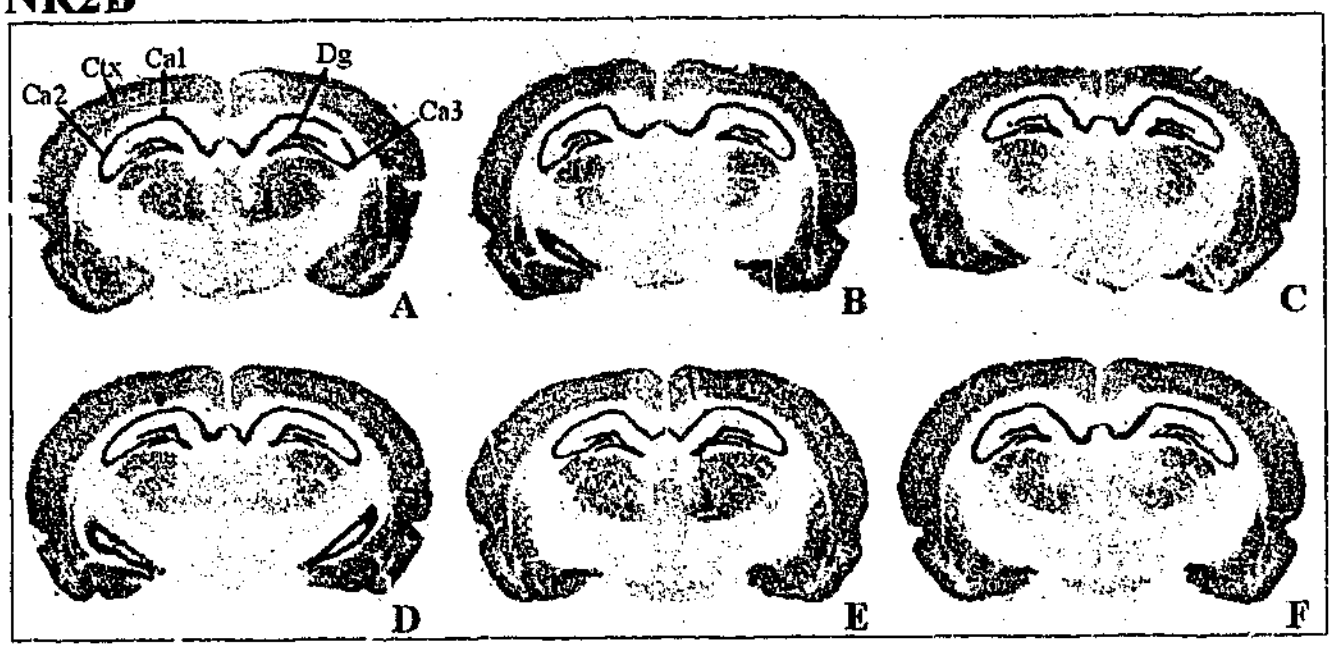


Figure 3.16. NR2B subunit mRNA expression in representative coronal sections 3.6 mm caudal to bregma from vehicle (A), 300 pmol/day β-amyloid$_{(1-40)}$ (B), 0.75 mg/kg/day nicotine (C), 300 pmol/day β-amyloid$_{(1-40)}$ + 0.75 mg/kg/day nicotine (D), 1.25 mg/kg/day chronic nicotine (E) and 300 pmol/day β-amyloid$_{(1-40)}$ + 1.25 mg/kg/day nicotine treated animals. Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus.

The levels of mRNA expression for the NR1, NR2A and NR2B NMDAR subunits in each treatment group, measured in terms of relative optical density, are shown in figure's 3.17, 3.18 and 3.19 respectively. Chronic i.c.v. infusion of β-amyloid did not effect the level of mRNA expression of any of these subunits. Chronic nicotine treatment had no effect on NMDAR subunit mRNA expression levels at a dose of 0.75 mg/kg/day but caused a significant decrease in the level of expression of the NR1 and NR2A subunits in the Ca3 area of the hippocampus and the cortex. Interestingly, there was no significant change in NR1 or NR2A mRNA expression levels in the treatment group that received both 1.25 mg/kg/day nicotine and chronic i.c.v. β-amyloid infusion. For the NR1 subunit the respective changes in Ca3 and cortical subunit mRNA expression for the 1.25 mg/kg/day chronic nicotine / 1.25 mg/kg/day chronic nicotine + β-amyloid infused groups were –14% / -4% and –22% / +3% relative to the vehicle treated group. For the NR2A subunit the respective changes in Ca3 and cortical subunit mRNA expression for the 1.25 mg/kg/day chronic nicotine / 1.25 mg/kg/day chronic nicotine + β-amyloid infused groups

were -25% / -16% and -44% / -16% relative to the vehicle treated group. The level of NR2B-NMDAR subunit mRNA expression was not affected by continuous β-amyloid infusion, chronic nicotine treatment or a combination of these treatments relative to vehicle treated rats.

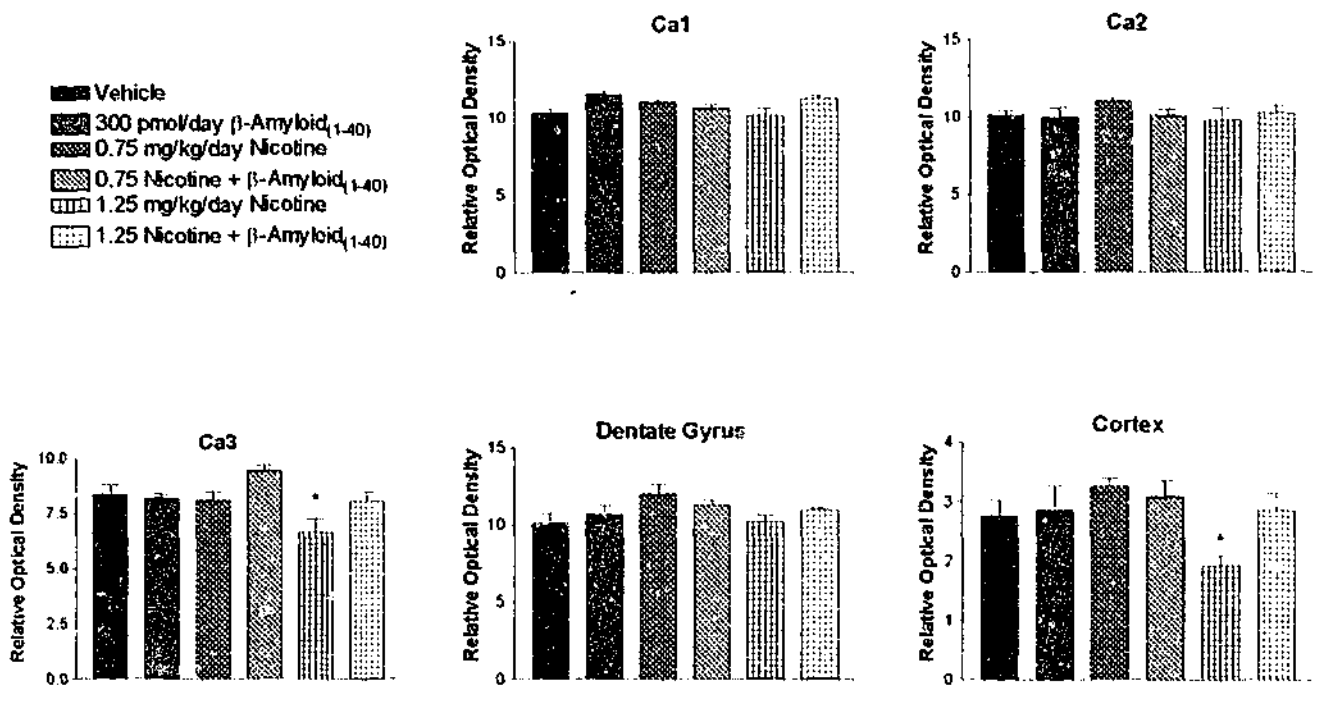


Figure 3.17. Levels of NR1 subunit mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus. The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post-test (*, $P < 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

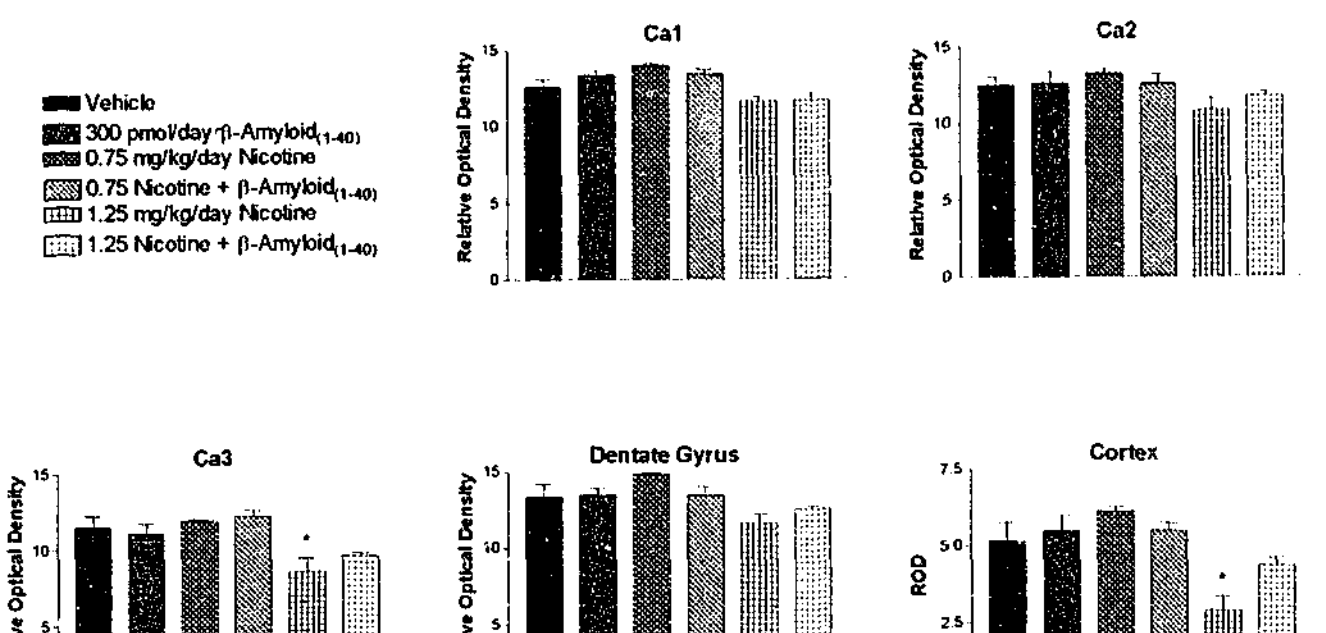


Figure 3.18. Levels of NR2A subunit mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus. The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post-test (*, $P < 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

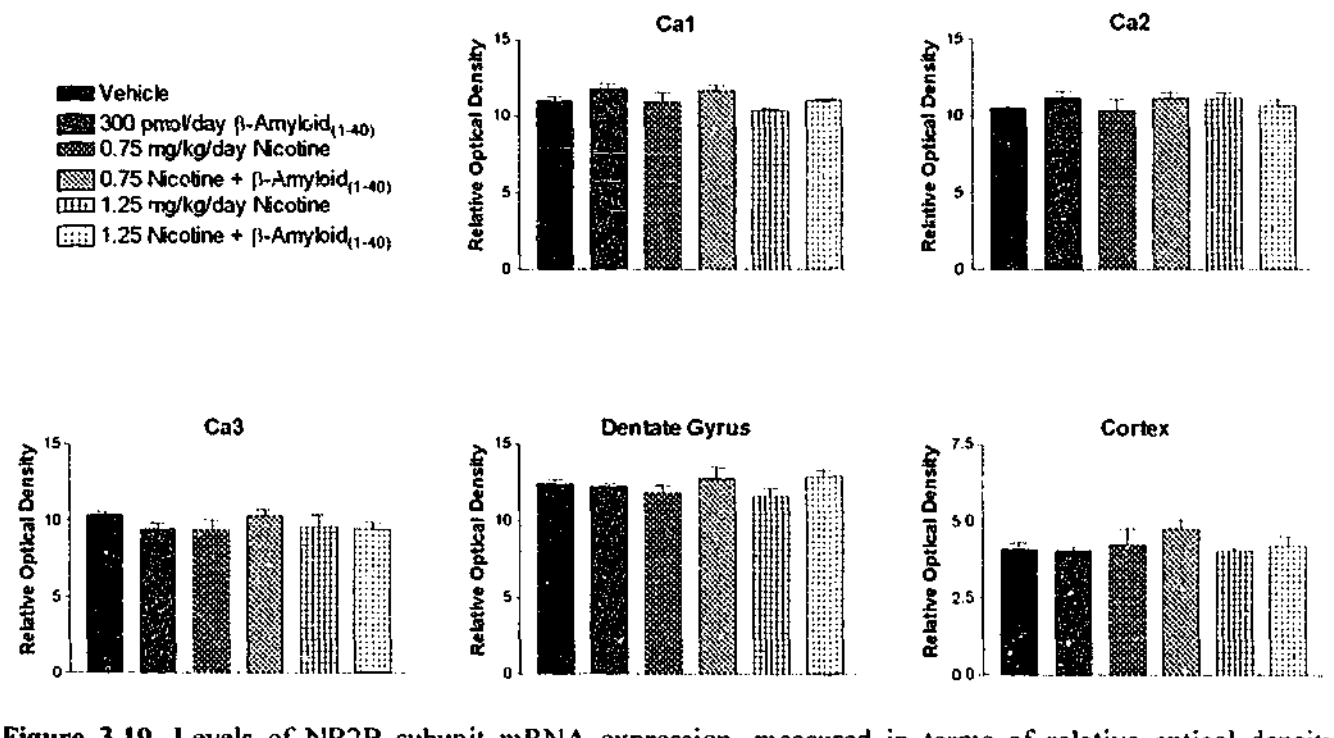


Figure 3.19. Levels of NR2B subunit mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus. The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post test. Differences in the means were not statistically significant in all cases ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

3.3.8 APP695, BACE, PS1, PS2 AND TACE MRNA EXPRESSION

The mRNA expression of APP695 and four proteins (BACE, PS1, PS2 and TACE) that play critical roles in the proteolytic processing of APP was examined in each treatment group using *in situ* hybridisation histochemistry. The distribution of APP695, BACE, PS1, PS2 and TACE mRNA in representative coronal sections is shown below in figure 3.20. The time of film exposure required to generate an autoradiographic image gives a qualitative indication of the relative abundance of a particular mRNA. APP695 mRNA, which required only 3 days of film exposure, is seen at very high levels throughout the hippocampus and cortex and is particularly prominent in cortical layer 3 (as defined in the atlas of Paxinos and Watson (1986). BACE and PS1 mRNA images were generated after 2 weeks and both showed relatively high levels of expression throughout the hippocampus and cortex. PS2 and TACE mRNAs, however, are much less prominent in the rat brain and 5 weeks on film was required before adequate autoradiographic images were generated. PS2 mRNA is expressed in the hippocampus and at low levels in the cortex. TACE mRNA is expressed at very low levels in the rat brain.

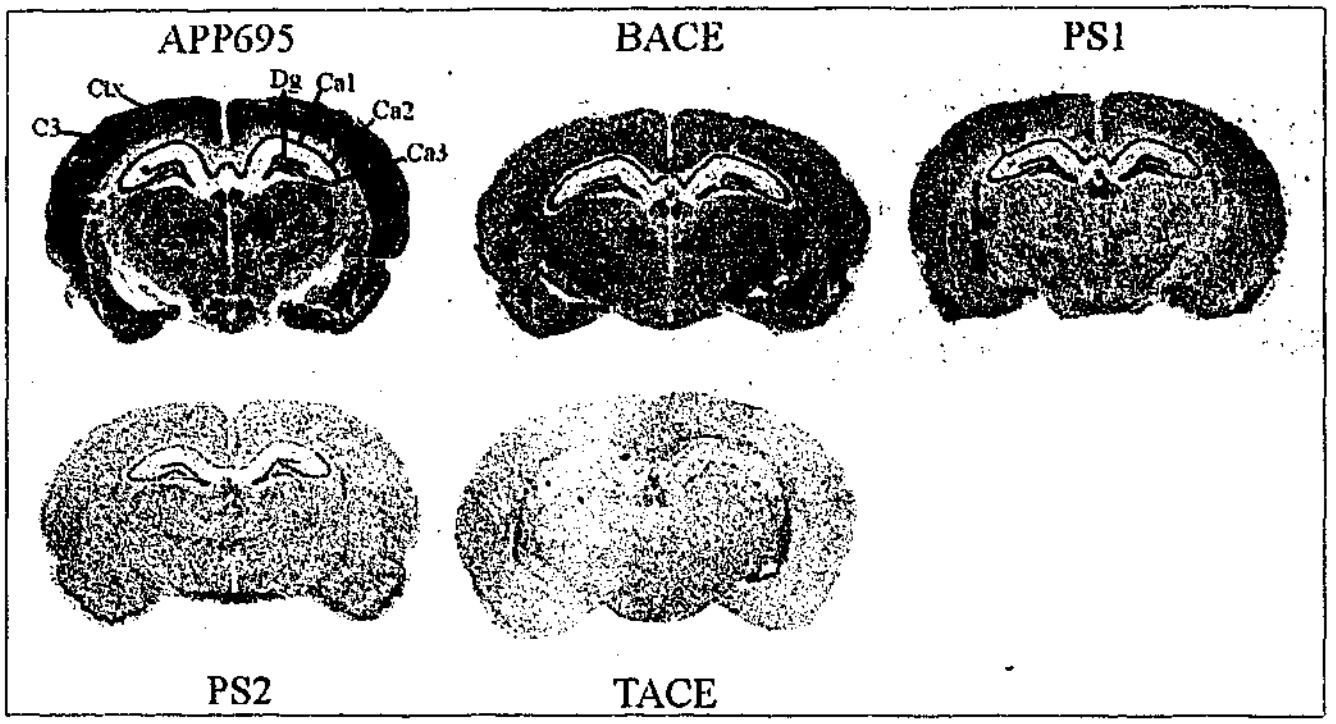


Figure 3.20. Distribution of APP695, β-site APP-cleaving enzyme (BACE), presenilin-1 (PS1), presenilin-2 (PS2) and tumour necrosis factor-α-converting enzyme (TACE) mRNA in representative coronal sections approximately 3.6 mm caudal to bregma. Abbreviations: Ca1-3, fields of the hippocampus; C3, cortical layer 3; ctx, cortex; Dg, dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Densiometric analysis of images from each treatment group revealed that APP695, BACE, PS1, PS2 or TACE mRNA expression was not significantly affected by 14-day continuous i.c.v. Aβ infusion, 21-day chronic nicotine (0.75 or 1.25 mg/kg/day) exposure, or a combination of these treatments relative to vehicle treated animals ($P > 0.05$, one way ANOVA with a Tuckey post-test for multiple comparisons; fig.'s. 3.21 – 3.25).

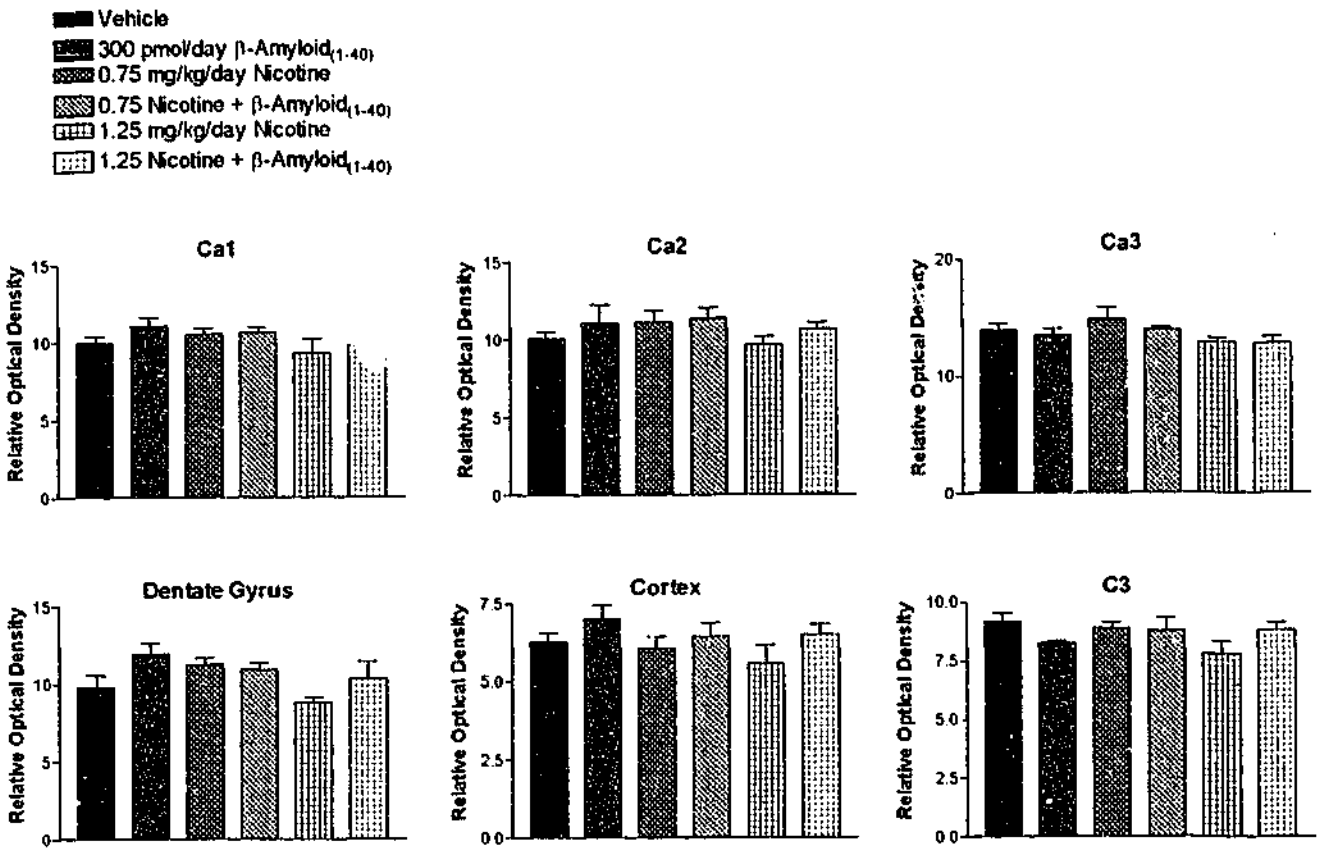


Figure 3.21. Levels of APP695 mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus; C3, cortical layer 3. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post test. Differences in the means were not statistically significant in all cases ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

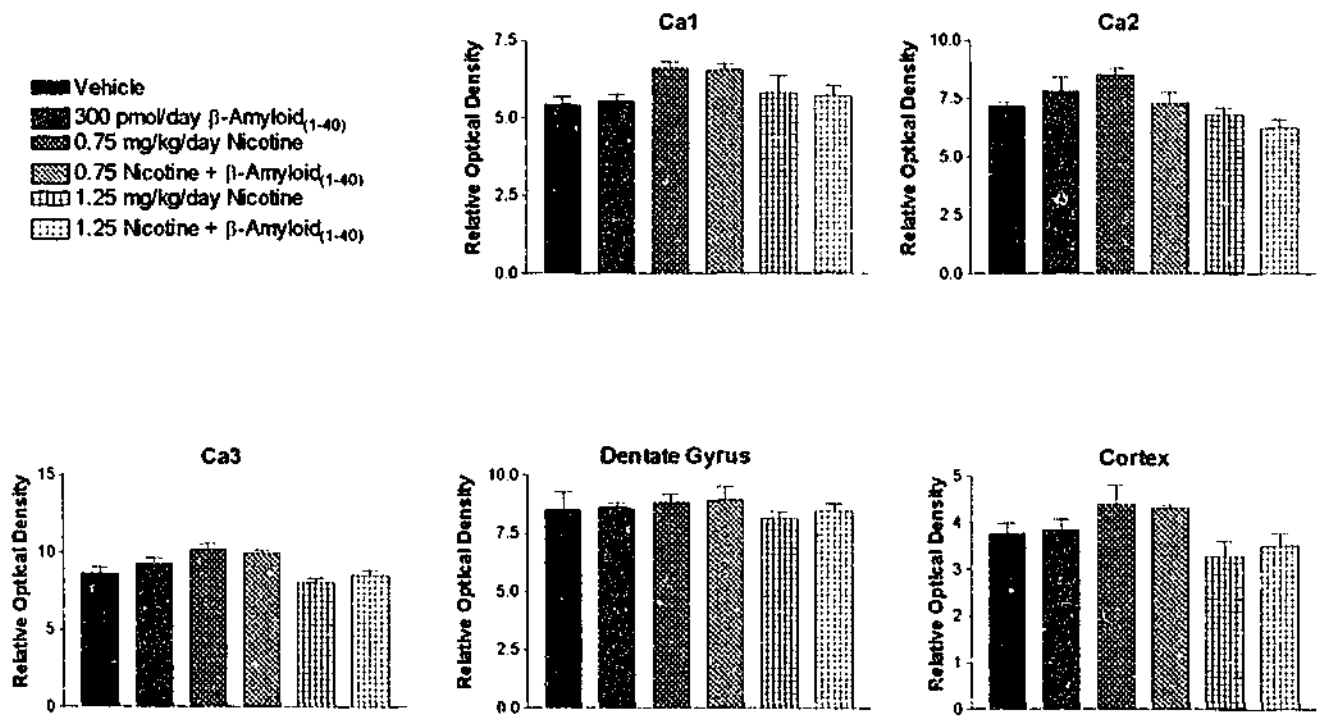


Figure 3.22. Levels of β-site APP-cleaving enzyme (BACE) mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus. The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post test. Differences in the means were not statistically significant in all cases ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

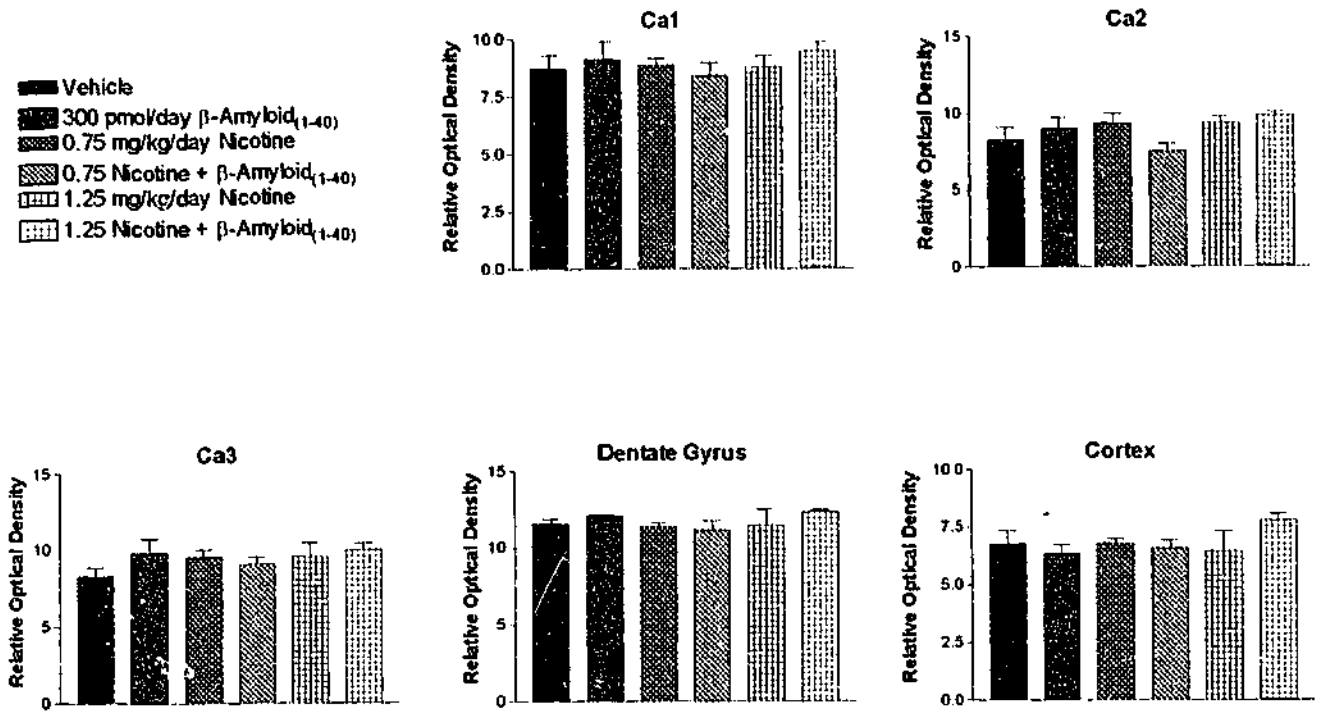


Figure 3.23. Levels of presenilin-1 (PS1) mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus. The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post test. Differences in the means were not statistically significant in all cases ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

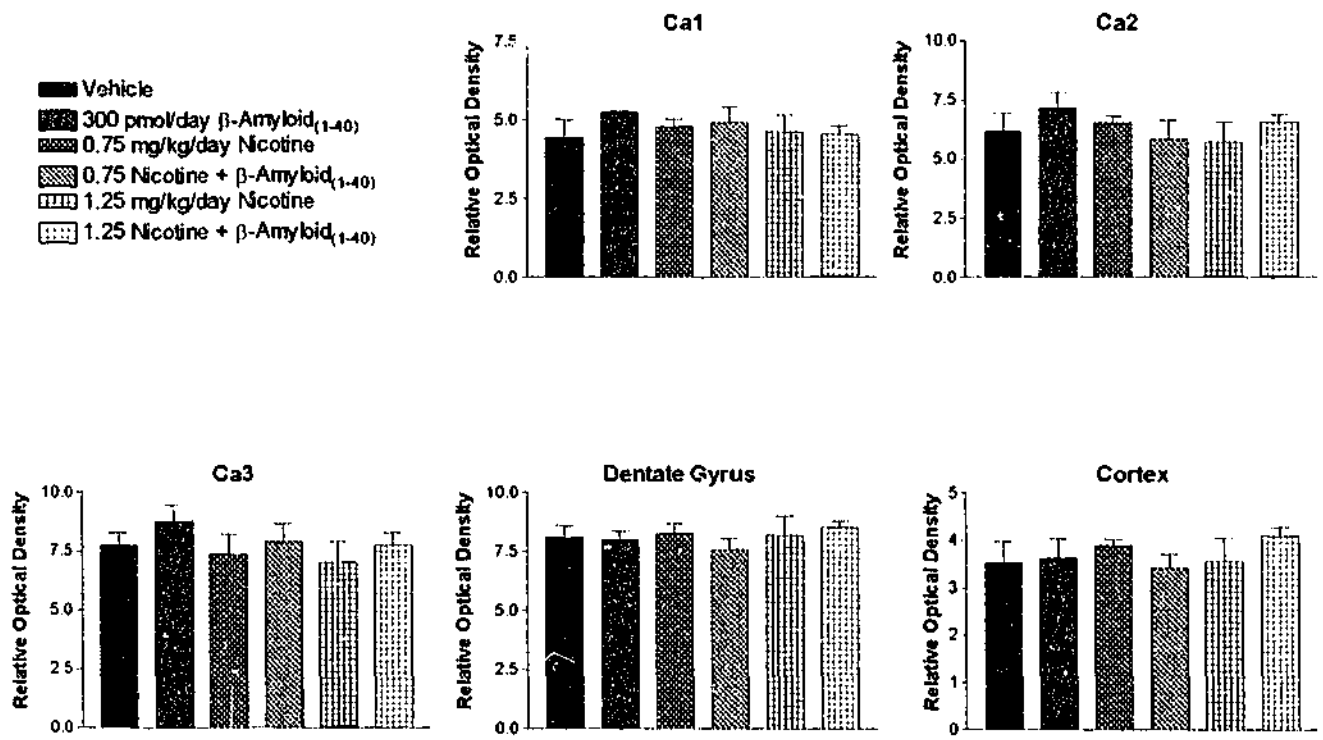


Figure 3.24. Levels of presenilin-2 (PS2) mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus. The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post test. Differences in the means were not statistically significant in all cases ($P > 0.05$ Each data point represents the mean ± S.E.M. of measurements taken from four animals.

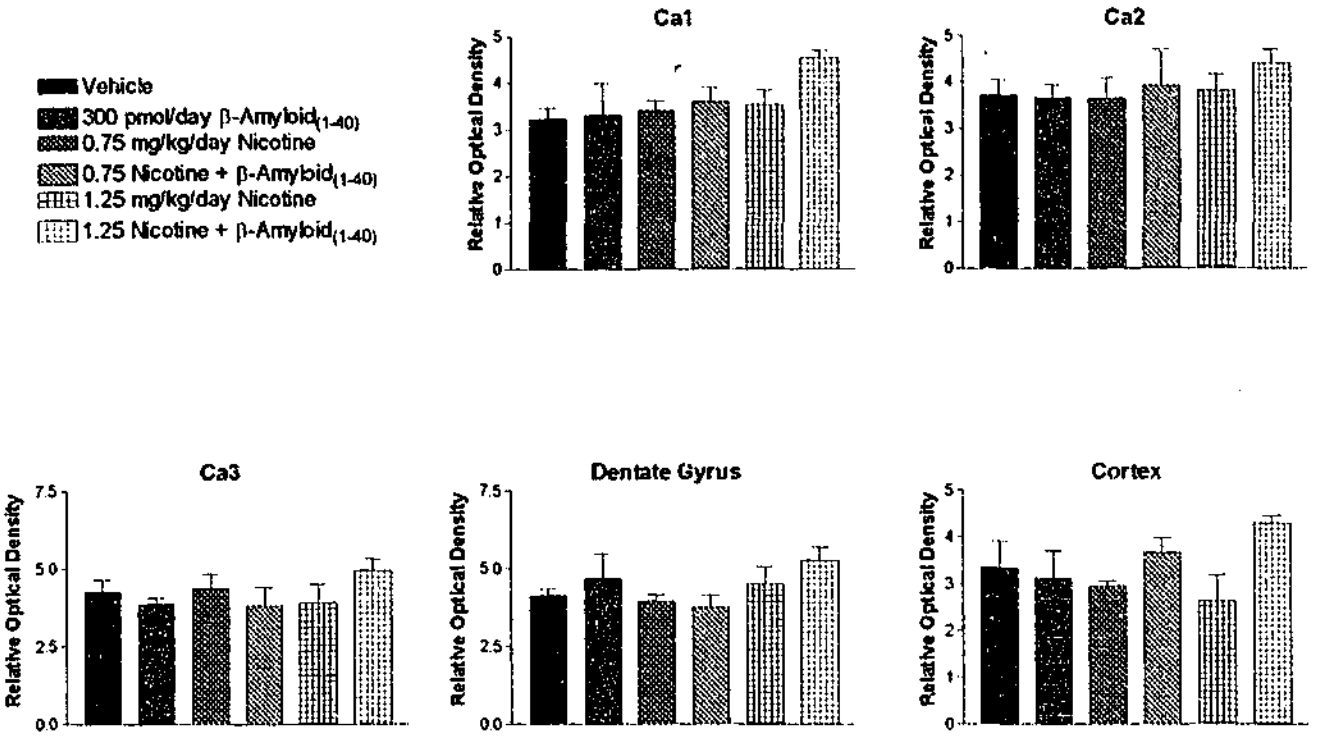


Figure 3.25. Levels of tumour necrosis factor-α-converting enzyme (TACE) mRNA expression, measured in terms of relative optical density (ROD), in selected regions of the rat brain following 21-day chronic nicotine treatment, 14-day continuous infusion of β-amyloid$_{(1-40)}$ or a combination of these treatments. Abbreviations: Ca1-3, fields of the hippocampus. The mean ROD for the vehicle group was compared to the mean ROD for each treatment group using one-way ANOVA with a Tuckey post test. Differences in the means were not statistically significant in all cases ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

3.4 DISCUSSION

The hypothesis that the neurodegenerative process associated with AD may be mediated by the direct neurotoxic actions of Aβ peptides remains a controversial one in AD research. Under the conditions employed in this study there was no evidence to suggest a neurotoxic mechanism of action for soluble $A\beta_{(1-40)}$ *in vivo*, in relation to cholinergic neurons at least, as assessed by ChAT activity and AChE levels. This finding is consistent with other studies that report a lack of neurotoxic affect following *in vivo* Aβ peptide treatment (Abe et al., 1994; Cole et al., 1997; Games et al., 1992; Winkler et al., 1994). However, these results do not support previously reported findings describing the neurotoxic properties of Aβ peptides *in vitro* (Mattson et al., 1992; Pike et al., 1993; Yanker et al., 1990). There are a number of possible reasons for this. Perhaps the simplest explanation is that the neurotoxic properties of Aβ peptides are critically dependent on the length and aggregation state of the Aβ fragment and/or factors that vary between *in vitro* and *in vivo* preparations. Most *in vitro* studies have required the use of pre-aggregated forms of the $A\beta_{(1-42)}$, as apposed to the soluble $A\beta_{(1-40)}$ peptide used here, in order to demonstrate a neurotoxic affect (e.g. Pike et al., 1993). It is also notable that most of these experiments have used immature neuronal cell cultures derived from embryonic or early postnatal animals. These protocols therefore have limited relevance to physiologically integrated systems, particularly when one considers AD is an age related disorder. Furthermore, the concentrations of Aβ required for neurotoxicity *in vitro* are as much as 10 000-fold higher than the concentration of total Aβ found in the CSF of AD patients (Neve et al., 1998). The amount of Aβ used in the present study (300 pmol/day) is more representative of the picomolar concentrations of $A\beta_{(1-40)}$ found in CSF from both AD and

control subjects (Ida et al., 1996; Seubert et al., 1992; Vigo-Pelfrey et al., 1993). In regard to previous *in vivo* studies, both Nitta et al., (1997) and Yamada et al., (1998) have reported a significant reduction in ChAT activity in the hippocampus following $A\beta_{(1-40)}$ treatment under similar conditions to those used in the present study. While difficult to explain, this discrepancy is perhaps not surprising in light of the relatively modest changes in ChAT activity reported by Nitta *et al* (1997) and Yamada *et al* (1998) and when one considers the significant amount of variation in the literature in relation to the neurotoxic affect of Aβ peptides *in vivo*. It should be noted that such variation is probably attributable to some extent to the wide range of methodologies used to study the affects of Aβ peptides *in vivo* (refer to table 3.1, *3.1 Introduction*). Major differences in the various protocols used include variations in the length (12-28, 25-35, 1-40, 1-42), conformation (aggregated or soluble), delivery (single injection or continuous infusion) and duration of action of the Aβ peptide. Different protocols may be important in modelling different stages of the disease process in relation to Aβ accumulation. On balance, it seems any neurotoxic effects mediated by Aβ peptides are more likely to be associated with the longer, fibrogenic $A\beta_{(1-42)}$ peptide rather then the more soluble $A\beta_{(1-40)}$ isoform. Generally, direct injection of aggregated Aβ peptides into parenchymal tissue has been the most consistent way to demonstrate a neurotoxic effect (e.g. Emre et al., 1992; Harkany et al., 2000b; Pepeu et al., 1996). Such methods usually result in dense deposits of Aβ similar to the neuritic plaques seen in latter stages of AD. On the other hand, infusions of soluble Aβ peptides, as has been used in this study, result in a more diffuse distribution of Aβ with very few focal plaque-like amyloid deposits (Frautschy et al., 1996), similar to the morphology seen in the earlier stages of AD that precede neuritic plaque formation. A number of studies report that in elderly non-demented individuals, an increase in the level of Aβ in the form of diffuse plaques predates neurofibrillary pathology and clinical dementia (Coria et al., 1993; Crystal et al., 1988; Morris et al., 1996). The brains of these individuals lack significant

neocortical neuronal degeneration. These clinical observations are consistent with the results reported here, which also show that a significant elevation in the level of soluble Aβ is not necessarily associated with the death of cholinergic neurons. It has been suggested that the accumulation of Aβ in the absence of neurodegeneration or neurofibrillary pathology may be representative of a 'preclinical stage' in AD (Vickers et al., 2000). Therefore, the continuous infusion of soluble Aβ peptides (as apposed to parenchymal injections of aggregated forms of Aβ, which is associated with neuronal death) may be a useful model with particular relevance to early stages of the neurodegenerative process associated with AD. Interestingly, a study by (Morris et al., 1996) demonstrated that aged, non-demented individuals who exhibit neocortical Aβ deposits do show specific minor cognitive impairments indicative of an early stage of AD. As discussed below, it is possible that certain *neuromodulatory* rather than neurotoxic actions mediated by Aβ peptides may underlie this effect.

A common misconception regarding Aβ peptides is that they are only produced as a result of abnormal, amyloidogenic processing of APP. It is important to remember that $A\beta_{(1\text{-}40)}$ and $A\beta_{(1\text{-}42)}$ are normal products of APP metabolism and both are found in CSF in similar concentrations in both normal AD subjects (Ida et al., 1996). This raises the possibility that Aβ peptides may be physiologically active proteins with specific functions in the CNS. While no specific physiological role has been established, there is an increasing amount of evidence that suggests Aβ peptides may act as modulators of cholinergic neuron activity (for review see Auld et al., 1998). A number of laboratories have reported that Aβ peptides can potently induce cholinergic hypofunction *in vitro* (Kar et al., 1996; Kelly et al., 1996; Pedersen et al., 1996). Considering the importance of cerebral cholinergic activity in relation to both cognitive function and AD pathology (for reviews see (Bartus et al., 1982; Coyle et al., 1983; Fibiger, 1991; Robbins et al., 1997; Lawrence et al., 1998), it is certainly possible that modulation of cholinergic activity by

Aβ may underlie the cognitive impairment associated with AD. Kar et al., (1996) have demonstrated that picomolar to nanomolar concentrations of various Aβ fragments, including $A\beta_{(1-40)}$, can inhibit stimulated release of ACh from cortical and hippocampal slices. In support of this finding, a significant reduction in ACh release has also been observed following treatment with Aβ peptides *in vivo* (Abe et al., 1994). It has been suggested that limited availability of choline due to Aβ mediated inhibition of HACU may be the mechanism underlying the reduced ACh release following Aβ treatment (Auld et al., 1998). Kar et al., (1998) have reported that picomolar to nanomolar concentrations of $A\beta_{(1-40)}$, but not the reverse sequence ($A\beta_{(40-1)}$), significantly inhibits HACU in a dose dependent manner in hippocampal synaptosomes. This result is consistent with the findings reported in this chapter showing that $A\beta_{(1-40)}$, at a physiologically relevant dose, inhibits the binding of [H^3]HC-3 to HACU sites. Furthermore, the results presented here suggest that the inhibition of HACU reported by (Kar et al., 1998) may be due to a direct interaction of Aβ peptides with the HACU site. Nakamura et al., (2001) have recently reported that the longer $A\beta_{(1-42)}$ peptide also inhibits binding of [H^3]HC-3 to HACU sites. Interestingly, the work presented in this chapter as well as that conducted by Kar *et al* (1998) and Nakamura *et al* (2001) demonstrates that HACU is affected in the absence of any affect on ChAT activity. These results suggest that Aβ peptides may modulate the function of cholinergic neurons prior to, or independently of, any apparent neurotoxicity. Speculatively, this scenario may be representative of the aforementioned 'preclinical' stage of AD associated with increased Aβ levels and minor cognitive impairments in the absence of detectable neurodegeneration. Following extended exposure to $A\beta_{(1-42)}$, however, Nakamura *et al* (2001) have demonstrated that ChAT activity is eventually reduced in addition to an initial reduction in [^{3}H]HC-3 binding. This is consistent with clinical findings that show ChAT activity to be relatively unaffected in early stages of the disease (Davis et al., 1999) but

significantly reduced at later stages (Bowen et al., 1976; Davies et al., 1976; Perry et al., 1977).

Perhaps the most interesting result reported in this chapter concerns the effects of chronic nicotine treatment and Aβ treatment in combination with chronic nicotine treatment on [^{3}H]HC-3 binding to HACU sites. The finding that chronic nicotine exposure causes a reduction in [^{3}H]HC-3 sensitive HACU sites in a dose dependent manner has not been previously reported so far as can be determined. This effect is consistent with the idea that neurons may down-regulate the uptake of their transmitters in response to excessive neuronal stimulation (Gonzalez et al., 2000). Possibly, down-regulation of HACU sites represents a homeostatic response to sustained cholinergic stimulation associated with chronic nicotine treatment. The finding that co-treatment with both chronic nicotine and $A\beta_{(1-40)}$ causes a significantly greater reduction in [^{3}H]HC-3 binding than that associated with either $A\beta_{(1-40)}$ treatment or chronic nicotine exposure alone is an interesting result. Relative to the vehicle group, the degree of reduction in [^{3}H]HC-3 binding in the $A\beta_{(1-40)}$ + 1.25 mg/kg/day chronic nicotine group is greater than the sum of the reductions seen in the $A\beta_{(1-40)}$ or 1.25 mg/kg/day chronic nicotine groups alone. One may speculate that there is a synergistic rather than simply additive effect. Furthermore, in certain regions such as the granular layer of the dentate gyrus and cortex, while there was no significant change in [^{3}H]HC-3 binding associated with $A\beta_{(1-40)}$ or 0.75 mg/kg/day chronic nicotine treatment alone, co-treatment with both $A\beta_{(1-40)}$ and 0.75 mg/kg/day chronic nicotine caused a significant reduction in [^{3}H]HC-3 binding. Any potentiation by chronic nicotine exposure of a Aβ mediated modulation of cholinergic neuron function may be of significant relevance to smokers. Particularly if one considers the possibility that modulation of cholinergic function, specifically inhibition of HACU, may have pathological consequences. It has previously been hypothesised that choline deprivation may contribute to the cholinergic vulnerability seen in AD (Wurtman, 1992). In response to choline

deficiency, neuronal death may occur as a result of the metabolism of membrane bound phosphatidylcholine as an alternate choline source (Parducz et al., 1976; Ulus et al., 1989) (for review see Auld et al., 1998). This possibility is consistent with the previously mentioned study by Nakamura *et al* (2001), showing that a reduction in HACU sites precedes a loss of ChAT activity. Speculatively, any reduction in HACU associated with chronic nicotine exposure may exacerbate any Aβ mediated neurotoxic effect related to choline deprivation. This idea is consistent with recent studies which report an increase in the risk of AD associated with smoking (Merchant et al., 1999; Ott et al., 1998); for review see Kukull, 2001) and does not support epidemiological data that positively correlates smoking with a delayed onset of AD (for review see Fratiglioni et al., 2000). It should be noted that although the nicotine doses used in this study are similar to those seen in smokers, the continuous delivery regime used here is clearly different to the intermittent nicotine intake experienced by smokers.

It was interesting to find that the $\alpha_4\beta_2$ and α_7 nAChR levels or the expression of the corresponding subunit mRNAs were not significantly affected following continuous Aβ treatment. This result suggests that the consistent loss of these receptors seen in AD may not be related specifically to the accumulation of Aβ, at least in the $A\beta_{(1-40)}$ form, and is consistent with a study by Wevers et al., (1999) which reports that in AD brains there is no correlation between the amount of Aβ accumulation and the density of α_7 or α_4 receptor subunit proteins. These results are important in that they indicate that Aβ accumulation is not likely to be a causative factor in relation to the loss in nAChR density seen in AD. The increase in [^{3}H]epibatidine binding following chronic nicotine reported here is consistent with well documented findings that $\alpha_4\beta_2$ nicotinic receptors are up-regulated in response to chronic nicotine treatment (e.g. (Abdulla et al., 1995; Flores et al., 1997; Hsu et al., 1996; Rowell et al., 1997). The lack of any change in the expression of the corresponding subunits is not surprising in light of reports that receptor levels are likely to be regulated at

a post-transcriptional level rather then through mRNA expression (Marks et al., 1992; Pauly et al., 1996). The lack of change in the density of [^{125}I]α-BGT binding or α_7 subunit mRNA expression following chronic nicotine is also not surprising considering the likely cerebral concentrations achieved (~200-430 nM; interpolated from data presented in table 2.3) in the context of the affinity of (–)-nicotine for the α_7 receptor subtype in rats (5 μM). It is interesting to note that in the molecular layer of the dentate gyrus only the groups which received both chronic nicotine and Aβ exhibited an up-regulation in [^{3}H]epibatidine binding. This parallels well with the [^{3}H]HC-3 results which show that [^{3}H]HC-3 binding is not effected in the Molecular layer of the dentate gyrus following $A\beta_{(1-40)}$ or chronic nicotine treatment alone, but is significantly reduced as a result of a combination of these treatments. Notably, in all areas quantified the greatest changes in either [^{3}H]HC-3 or [^{3}H]epibatidine binding were consistently seen in the treatment group which received both $A\beta_{(1-40)}$ and 1.25 mg/kg/day chronic nicotine treatment. This was also the case in relation to changes in the expression of α_7 nAChR subunit mRNA. The only statistically significant change in α_7 nAChR subunit mRNA expression, was seen in the $A\beta_{(1-40)}$ + 1.25 mg/kg/day chronic nicotine treatment group as an increase in expression relative to the vehicle treated group. Collectively, the data presented in this chapter suggests that chronic nicotine exposure may potentiate any modulation of cholinergic neuron function mediated by Aβ peptides. Based on the affinity constants for (–)-nicotine at $\alpha_4\beta_2$ and α_7 nAChRs in rat brain, it is likely that activation of $\alpha_4\beta_2$, rather then α_7 receptors, underlies this effect. However, further experiments using antagonists are required before it can be established whether or not the effect of nicotine in these experiments is receptor mediated and which receptor subtypes are involved. Furthermore, it is worth noting that at the doses of nicotine used in this study the issue of receptor desensitisation is certainly relevant. A number of authors have reported that the EC_{50} for nicotine induced desensitisation in relation to its action as a modulator of transmitter release is ~10 nM with maximal desensitisation

produced at ~ 50 nM nicotine (Grady et al., 1994; Rowell et al., 1994). Based on the likely cerebral concentrations of nicotine achieved in this study (~200-430 nM), it is possible that the reported affects of chronic nicotine are attributable, to some extent, to receptor desensitisation.

Chronic nicotine treatment at the higher dose of 1.25 mg/kg/day resulted in a significant reduction in the expression of mRNA for the NR1 and NR2A NMDAR subunits in the cortex and Ca3 area of the hippocampus. This finding is consistent with the report that chronic (–)-nicotine treatment causes a significant reduction in the level of binding of the NMDAR selective agonist [^{3}H]MK-801 (Zhang et al., 1994b). Furthermore, the results presented here suggest that this reduction in NMDAR receptor density may be mediated at the receptor subunit mRNA level. It has previously been demonstrated that a reduction in NMDAR subunit mRNA is associated with a reduction in the levels of the corresponding subunit protein (Cebers et al., 2001). Speculatively, the down-regulation of NR1 and NR2A but not NR2B receptor subunits in the same anatomical regions seen here may represent a specific affect of chronic nicotine on an NMDAR subtype containing the NR1 and NR2A subunits. Also in support of the findings presented here, nanomolar concentrations of nicotine have been shown to stimulate glutamatergic neurotransmission (McGehee et al., 1995a) and increases in glutamate concentration can cause a significant reduction in the expression of multiple NMDAR subunit mRNAs in primary neuronal culture, including the NR1, NR2A and NR2B subunits (Cebers et al., 2001). It has been reported that nicotine interacts directly with the NMDAR based on findings that nicotine can displace [^{3}H]MK-801 binding in rat membranes (Aizenman et al., 1991). The reduction in NMDAR subunit mRNA associated with chronic nicotine treatment seen here may therefore represent a homeostatic response to excessive NMDAR stimulation through either nicotine stimulated glutamate release via presynaptic nAChRs, nicotine activating NMDARs directly or a combination of these events.

Chronic exposure to the $A\beta_{(1-40)}$ peptide did not significantly affect the level of expression of any of the NMDAR subunit mRNAs examined in this study. This finding is consistent with the report that there is no change in NMDAR levels in transgenic mice which exhibit significantly increased cerebral Aβ levels and AD-like neuropathology as a result of over-expression of a mutated (Swedish double mutation) form of human APP (Cha et al., 2001). It is interesting to note that while the 1.25 mg/kg/day chronic nicotine group showed a significant reduction in NMDAR subunit mRNA expression, this effect was not seen in the group which received $A\beta_{(1-40)}$ infusions in addition to the 1.25 mg/kg day chronic nicotine treatment. A study by Zhang et al., (1994b) reports that while chronic nicotine treatment causes a reduction in NMDAR density as determined by [^{3}H]MK-801 binding, this effect is prevented under conditions of significant NMDAR stimulation. In the context of the results presented here, this raises the interesting, albeit speculative, possibility that the $A\beta_{(1-40)}$ in the Aβ + chronic nicotine group causes NMDAR activation thereby preventing the reduction in NMDAR subunit mRNA expression seen as a result of chronic nicotine alone. As a direction for further work in this area, it would certainly be interesting to further investigate the possibility that Aβ peptides can directly activate NMDARs. Such an interaction may underlie the hypersensitivity to NMDA mediated excitotoxicity caused by treatment of neuronal cultures with Aβ peptides (Gray et al., 1995; Koh et al., 1990; Patel, 1995a).

A common phenomenon in biological science is that of positive and negative feedback loops whereby the products of a biological process affect the efficacy of the process itself. There is an extensive amount of literature describing feedback loops for receptor-neurotransmitter systems (e.g. Re, 1999) as well as for enzyme catalysed reactions (e.g. Jesty et al., 1993). It seems reasonable to hypothesise, therefore, that the proteolytic products of APP processing may affect the further processing of APP. One mechanism for such an effect may involve altered levels of expression of APP itself or the various proteins

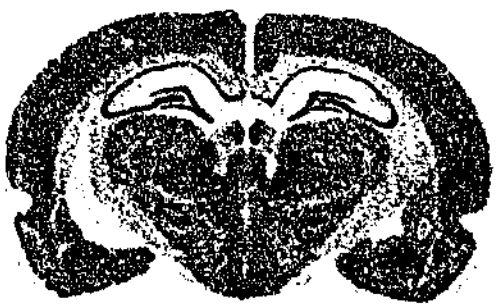
required for its proteolytic processing. It has previously been reported that, in addition to a significant increase in the level of $A\beta_{(1-40)}$ in AD brains (Mehta et al., 2000), there is also a significant reduction in PS1 and PS2 mRNA (Takami et al., 1997) as well as APP (Davidsson et al., 2001) levels compared to age-matched controls. It has been reported here however, relative to the vehicle treated group, a continuous 14-day i.c.v. infusion of $A\beta_{(1-40)}$ did not significantly affect the level of expression of APP695, BACE, PS1, PS2 or TACE mRNA. This result indicates that the significant increase in the level of $A\beta_{(1-40)}$ seen in AD brains, is not likely to be a causative factor in the change in PS1/2 and APP levels also seen in these subjects. It is of course possible that changes in the activity of the α–, β– and γ–secretase enzymes are mediated at a post-transcriptional level, and therefore not detected by the technique of *in situ* hybridisation histochemistry used here. As a direction for further work in this area, it would be interesting to examine the effect of chronic $A\beta_{(1-40)}$ exposure on other products of APP processing, such as sAPPα/β or p3, as a measure of secretase activity. It would also be worthwhile examining how chronic exposure of these other proteolytic products affects APP, BACE, TACE, PS1 and PS2 levels as well as secretase activity.

A number of studies have shown that cholinergic mechanisms play an important role in the stimulated processing of APP (for review see Rossner et al., 1998). While much of the available literature details the ability of protein kinase C (PKC) coupled muscarinic receptors to stimulate APP processing (Nitsch et al., 1992; Pittel et al., 1996; for review see Rossner et al., 1997), a study by Kim et al., (1997) has reported that treatment of PC12 cells with nicotine can also facilitate APP processing. Based on the time-dependence of this affect (> 2 hr), it is possible that mechanisms such as mRNA transcription play an important role in mediating this effect. It is reported here, however, that chronic nicotine at a dose of either 0.75 or 1.25 mg/kg/day does not effect the mRNA expression of a number of proteins involved in the proteolytic processing of APP, including BACE, PS1, PS2 and

TACE. This lack of effect may relate to the subtypes of nAChRs activated by these nicotine doses. As previously discussed, based on the affinity of nicotine for the $\alpha_4\beta_2$ and α_7 nAChR subtypes, the doses used in this study would be expected to primarily activate $\alpha_4\beta_2$, rather than α_7 nAChRs for which nicotine has a relatively poor affinity. However, the nicotine mediated processing of APP reported by Kim et al., (1997), is a concentration dependent effect with relatively high concentrations of at least 50 μM required. It therefore seems likely, that activation of α_7 rather than $\alpha_4\beta_2$ nAChRs may be important in the regulation of APP processing. It would be worthwhile investigating the effect of higher doses of chronic nicotine treatment, or perhaps chronic treatment with α_7 selective agonists, with regard to the expression of proteins involved in APP processing such as BACE, PS1, PS2 and TACE.

Chapter 4

The effect of selective degeneration of the cholinergic basal forebrain system on multiple neurochemical markers in the rat brain



4.1 INTRODUCTION

During the progression of AD the brain develops a complicated pattern of degenerative changes that includes the degeneration, atrophy or dysfunction of specific cell groups. Specifically, degeneration of the cholinergic basal forebrain nuclei is widely believed to underlie the most important clinical feature of AD – cognitive impairment.

A significant loss in ChAT activity in AD was first reported by Bowen *et al* in 1976 and shortly after by Davies *et al* and Perry *et al* in 1977. A loss in cholinergic activity and, more specifically, loss of cholinergic innervation of the cortex and hippocampus is one of the most severe and consistently reported neurochemical features of AD (Beach et al., 1997; Bigl et al., 1987; Bowen et al., 1976; Bowen et al., 1982; Davies et al., 1976; Perry et al., 1977; Zubenko et al., 1989). Broadly, the major cholinergic systems in the mammalian brain can be divided into three main groups, including: striatal interneurons; the basal forebrain cholinergic nuclei, which project to various telencephic regions including the hippocampus and cortex; and mesopontine tegmental cholinergic nuclei, which project primarily to thalamic targets (fig 4.0; Wainer et al., 1990). The loss of cholinergic activity throughout the hippocampus and cortex in AD is a result of the selective degeneration of cholinergic basal forebrain nuclei (Allen et al., 1988; Coyle et al., 1983; McGeer et al., 1984; Rinne et al., 1987; Vogels et al., 1990; Whitehouse et al., 1982). Intrinsic glutamatergic (Flint et al., 1985; Lamour et al., 1982; Reine et al., 1992), and to a lesser extent GABAergic (Beaulieu et al., 1991), neurons in the hippocampus and cortex are likely to represent the major synaptic target for these cholinergic basal forebrain projections. The role of the cholinergic basal forebrain system in memory and learning has been well documented (Henderson, 1996; Price et al., 1993; Wenk, 1997) and the progressive loss of basal forebrain cholinergic neurons has been closely correlated with the

learning and memory deficits seen in AD (Bowen et al., 1976; DeKosky et al., 1990; Mann, 1994; McGeer et al., 1984; Neary et al., 1986; Perry et al., 1978; Wainer et al., 1990; for review see Nabeshima, 1993). This fundamental change in cholinergic neurotransmission is the basis for the 'cholinergic hypothesis' of AD. This hypothesis, first described in 1982, states that a loss of cholinergic neuronal function is a central pathogenic event in the progression of AD which contributes significantly to the impairment in memory and cognition associated with the disease (Bartus et al., 1982).

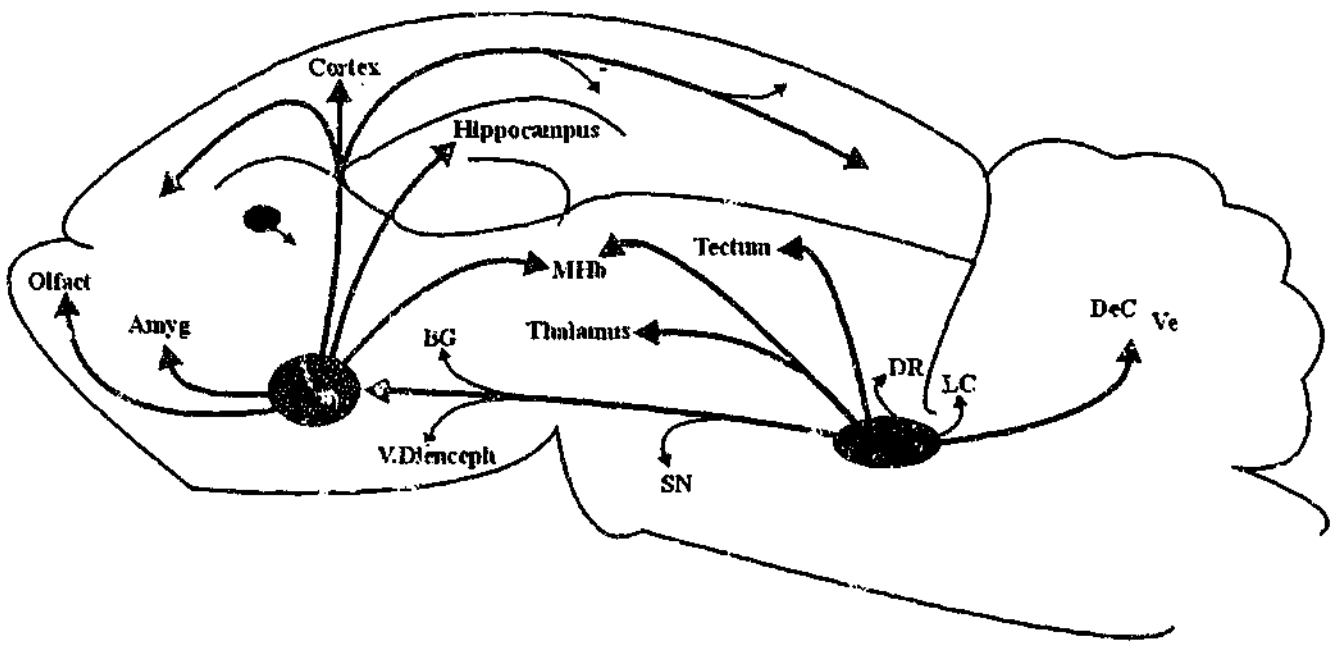


Figure 4.0. Schematic, parasagital representation of the major cholinergic systems in the mammalian brain. Central cholinergic neurons can be broadly classified as: anatomically localised interneurons, primarily located in the striatum (Si, ●); diffusely projecting basal forebrain nuclei (BF, ●); and diffusely projecting mesopontine tegmental nuclei (MPT, ●). The major ascending afferent pathways of the basal forebrain and mesopontine tegmental cholinergic systems are illustrated. Basal forebrain cholinergic neurons project extensively to the cortex and hippocampus (including - anterior cingulate, entorhinal, frontal, insular, parietal, perihinal, piriform, retrosplenial, temporal and visual cortical regions and the Ca1, Ca2, Ca3 and dentate gyrus regions of the hippocampus). Other cholinergic systems located in the spinal cord as well as skeletal motor and autonomic subdivisions of the cranial nerve have not been represented here. Abbreviations: Amyg, amygdaloid nuclei; BG, basal ganglia; DeC, deep cerebellar nuclei; DR, dorsal raphe nucleus; LC, locus ceruleus; MHb, medial habenula; Olfact, olfactory bulb; SN, substantia nigra; V.Diencph, ventral diencephalon; Ve, vestibular nuclei.

Recognition of the role of cholinergic dysfunction in the cognitive impairment associated with AD has led to the development of drugs that selectively enhance

cholinergic function by inhibition of the cholinergic catabolic enzyme, AChE. As previously discussed, there are a number of limitations associated with anticholinesterase therapy of AD, due largely to significant side effects, and also to the highly variable efficacy of AChE with regard to enhancement of cognitive function. To some degree this is probably related to the necessity of a certain level of existing cholinergic neurotransmission required for the beneficial action of anticholinesterases. In latter stages of AD, the profound loss of cholinergic activity may prevent further enhancement of cholinergic transmission by AChE inhibition. It would therefore be useful to identify alternative therapeutic targets for AD that persist throughout the progression of the disease and/or present fewer problems associated with side effects. Although there is a good deal of evidence which describes a correlation between loss of cholinergic neurodegeneration and cognitive disturbances in AD, the relationship between this loss of cholinergic function and changes in other neurochemical markers which may be important in memory and cognition, or the pathology of the disease, remains less clear. The aim of the work presented in this chapter has been to explore this relationship by investigating the effect of selective degeneration of the cholinergic basal forebrain neurons in the rat on other neurochemical markers known to be effected in AD. Specifically, the neurochemical markers examined include: nAChR binding and mRNA expression; NMDAR and neuronal nitric oxide synthase (nNOS) mRNA expression; and the expression of mRNAs encoding for APP695, BACE, PS1, PS2 and TACE. Although the relevance of each of these groups of neurochemical markers to AD have been discussed in Chapter 1, *General Introduction*, the focus of the introduction to this chapter has been to discuss how these markers may be related specifically to cerebral cholinergic function.

Selective lesioning of the cholinergic basal forebrain system was achieved using the immunotoxin, 192-IgG-saporin. The 192-IgG-saporin immunotoxin is a monoclonal antibody for the rat p75 (low affinity) nerve growth factor (NGF) receptor, conjugated via

disulphide bonds to the cytotoxic ribosome inactivating protein, 192-IgG-saporin. The toxin is selective for cholinergic basal forebrain neurons by virtue of the monoclonal antibody targeted to p75 NGF receptors. The cholinergic neurons of the basal forebrain are the principal site of NGF action in the CNS and are among the few neurons in the mature CNS that express the p75 NGF receptor (Batchelor et al., 1989; Pioro et al., 1990). After binding to p75 NGF receptors on the somata and axons of cholinergic basal forebrain neurons, internalisation of the receptors allows the 192-IgG-saporin access to ribosomes, halting protein synthesis and causing cell death. Both i.c.v. administration of 192-IgG-saporin as well as direct injection into basal forebrain nuclei causes a nearly complete and specific lesion of cholinergic cells in the basal forebrain, while sparing other cholinergic systems such as the mesopontine tegmental nuclei and striatal interneurons (Book et al., 1992; Heckers et al., 1994; Holley et al., 1994; Lee et al., 1994; Nilsson et al., 1992; Torres et al., 1994; Wenk et al., 1994). This is evidenced by a significant loss in cholinergic markers such as ChAT and AChE in areas innervated by cholinergic basal forebrain projections, including the hippocampus and cortex, but not in areas such as the striatum, cerebellum or most thalamic nuclei, which do not receive a significant level of cholinergic basal forebrain innervation (Book et al., 1995; Heckers et al., 1994; Rossner et al., 1995c). In terms of the relevance of this model to the clinical symptoms associated with AD, behavioural studies have demonstrated significant impairments in both spatial and non-spatial learning after cholinergic lesioning (Book et al., 1995; Heckers et al., 1994; Nilsson et al., 1992; Rossner et al., 1995c; Vnek et al., 1996; Wiley, 1992).

As discussed previously, the loss of nAChRs in AD and the clinical potential for nAChR agonists in relation to mnemonic and cognitive function have been well documented. It is perhaps surprising then that there is little evidence to suggest that the degeneration of basal forebrain cholinergic neurons and loss of nAChRs seen in AD are related events. In fact, at least two studies report that the loss of ChAT activity seen in the

brains of AD subjects relative to aged-matched controls is *not* correlated to changes in the level of [^{3}H]epibatidine binding (Sabbagh et al., 1998; Whitehouse et al., 1995). Furthermore, it has been reported that there is no change in the level of nAChR binding, including [^{3}H]nicotine, [^{3}H]cytisine and [^{3}H]epibatidine, following experimental lesioning of the cholinergic basal forebrain neurons in rats (Bednar et al., 1998; Rossner et al., 1995b). One of the aims of the work presented in this chapter has been to extend on these studies by not only looking at [^{3}H]epibatidine binding, but also examining [^{125}I]α-BGT binding as well as the expression of the α_4, β_2 and α_7 nAChR subunit mRNAs in rat brain at a number of time-points following selective immunotoxic lesioning of the cholinergic basal forebrain neurons. By its very nature, AD is a progressive disorder and therefore multiple neurochemical systems are differentially affected at different time-points during the progression of the disease (Braak et al., 1991). For example studies showing that ChAT activity is not affected early in AD (Davis et al., 1999), but are significantly reduced at later stages of the disease (Bowen et al., 1976; Davies et al., 1976; Perry et al., 1977), suggest that the degeneration of cholinergic neurons in AD is progressive in nature. Therefore, the work presented in this chapter has examined how various neurochemical markers are affected at number of time-points after treatment with the cholinergic immunotoxin, 192-IgG-saporin. Specifically, measurements were taken at 4, 7, 14, 30 and 90 days after treatment with 192-IgG-saporin.

Another neurotransmitter-receptor system that has been shown to play an important role in mnemonic and cognitive function is the glutamatergic NMDA receptor system. As discussed previously, a number of studies have indicated that glutamatergic neurotransmission becomes hypofunctional in AD (Gonzales et al., 1991; Magnusson et al., 1993; Saransaari et al., 1995; Wenk et al., 1991) and is associated with reductions in NMDAR density (Greenamyre et al., 1987; Greenamyre et al., 1985). Less is known, however, about how this effect might be related to changes in the mRNA expression of the

corresponding protein subunits. NMDA receptors mediate excitatory neurotransmission within the dentate gyrus and at principal neurons in the Ca3 and Ca1 fields of the hippocampus (Swanson et al., 1987) and are crucial for the induction of long term potentiation (LTP) of synaptic transmission in these areas (Collingridge, 1987). As discussed earlier, LTP is a form of synaptic plasticity thought to be an important mechanism underling the physiological basis of memory. A number of lines of evidence suggest that cholinergic basal forebrain transmission may modulate glutamatergic neurotransmission and NMDAR activation in the cortex and hippocampus. Stimulation of nAChRs has been shown to modulate glutamatergic transmission (McGehee et al., 1995a; Schilstrom et al., 2000) and specifically, activation of α_7 nAChRs on glutamatergic mossy fibres can regulate glutamate release in the hippocampus (Gray et al., 1996). Experimental lesions of the cholinergic basal forebrain using 192-IgG-saporin have resulted in either no change (Nicolle et al., 1997) or a significant decrease (Rossner et al., 1995a) in NMDAR levels in the hippocampus. Most of the studies which have investigated NMDAR pharmacology in response to 192-IgG-saporin treatment lie within a fairly narrow range of post-treatment time points (between 1-4 weeks), have generally examined hippocampal regions and have not quantitated NMDAR subunit mRNA expression. The aim of this chapter, in regard to NMDARs, has been to extend on these studies by examining the effect of selective basal forebrain cholinergic denervation on the cortical and hippocampal expression of the NR1, NR2A and NR2B NMDAR subunits at 4, 7, 14, 30 and 90 days following i.c.v. injections of 192-IgG-saporin.

A number of studies have indicated that nitric oxide (NO) is likely to play an important role in the LTP mediated by NMDAR activation (for reviews see Brenman et al., 1997; Holscher, 1997). The process of LTP describes the strengthening of synaptic connections between glutamatergic neurons where postsynaptic activation of NMDARs increases the probability of presynaptic transmitter release thereby enhancing the level of

neuronal transmission at that synapse over extended periods of time. For postsynaptic receptor activation to increase presynaptic transmitter release, a form of retrograde communication must exist. Nitric oxide is a lipophilic molecular mediator that readily diffuses across cellular membranes and there has been a considerable amount of work published which supports the role of NO as a retrograde messenger that facilitates LTP (Arancio et al., 1996; Bohme et al., 1991; Hindley et al., 1997; Mizutani et al., 1993; O'Dell et al., 1991; Southam et al., 1996). The proposed mechanism for NMDAR and NO mediated LTP is described in figure 4.1. The involvement of NO in various aspects of learning and memory has been reported by a number of studies that show learning and memory impairments can be induced by the inhibition of NO synthesis (Bohme et al., 1993; Chapman et al., 1992). Nitric oxide is generated from L-arginine and catalysed by a family of three NO synthase (NOS) enzymes. NOS from endothelial cells (eNOS) and neurons (nNOS) are both constitutively expressed, but depend on stimulation by increases in intracellular calcium ($[Ca^{2+}]_i$), while stimulation of inducible NOS (iNOS) is independent of $[Ca^{2+}]_i$.

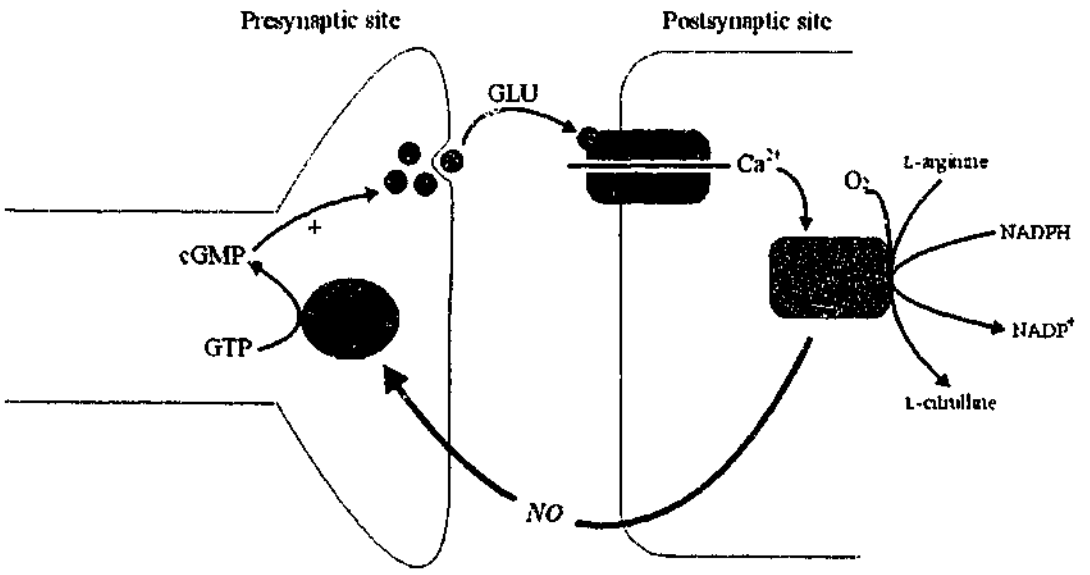


Figure 4.1. Schematic representation illustrating the proposed action of nitric oxide as a retrograde messenger in long term potentiation (LTP) of synaptic plasticity. Activation of NMDARs by glutamate causes an increase in postsynaptic $[Ca^{2+}]_i$ which activates nitric oxide synthase. NO then diffuses randomly through the cytosol and membranes and is absorbed by the haem group of a NO-sensitive guanylate cyclase. Production of cGMP then evokes an increase in transmitter release via an unknown mechanism, while transmitter reuptake is decreased. Abbreviations: cGMP, cyclic guanylate monophosphate; GC, guanylate cyclase; GLU, glutamate; GTP, guanylate triphosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NMDAR, N-methyl-D-aspartate receptor; NO, nitric oxide; NOS; nitric oxide synthase.

The results from a number of studies have implicated the involvement of NO, and in particular nNOS, in the pathogenesis of AD. Nitric oxide has been reported to possess neurotoxic properties and has been suggested to be involved in a number of neurodegenerative disorders (Dawson et al., 1991; Loiacono et al., 1992; Przedborski et al., 1996; Schulz et al., 1996). Thorns et al., (1998) have shown a significant loss of nNOS expressing neurons in the entorhinal cortex and, to a lesser extent, in the Ca1 and Ca3 fields of the hippocampus in patients with AD, while (Hyman et al., 1992) have shown a relative sparing of nNOS containing neurons in the hippocampal formation. Norris et al., (1996) have reported that while there is a significant decrease in the number of nNOS mRNA expressing neurons in AD brains, there is no significant change in the cellular level of nNOS mRNA within the existing neurons. The relationship between the reported changes in nNOS levels in the hippocampus and cortex and the loss of cholinergic innervation of these areas has not been extensively characterised. Stimulation of basal forebrain neurons elicits regional cerebral blood flow increases which are reportedly mediated by cholinergic stimulation of nNOS containing neurons (Adachi et al., 1992; Vaucher et al., 1997) and therefore there is some basis to suggest that nNOS activity in the cortex and hippocampus may be related to cholinergic basal forebrain activity. The aim of the work presented in this chapter, in relation to nNOS, has been to investigate how the expression nNOS mRNA in the cortex and hippocampus is affected at various time points, up to three months, after a loss of cholinergic basal forebrain activity.

In addition to regulating cortical and hippocampal blood flow, there is evidence to suggest that cholinergic activity may regulate the processing of APP in these areas. Experiments *in vitro* have shown that both muscarinic and nicotinic receptor stimulation may be important in APP processing. Muscarinic M_1 and M_3 receptor agonists are capable of stimulating the release of soluble APP from cortical slices in a dose dependent manner (Pittel et al., 1996), while nicotine has been shown to significantly increase the release of

soluble APP in neuronal cell culture and this affect can be blocked with the use of nAChR selective antagonists (Kim et al., 1997). Furthermore, Nitsch et al., (1993) has reported that electrically stimulated depolarisation in hippocampal slices causes an increase in soluble APP secretion that can be blocked by tetrodotoxin, indicating that this release results from formation of action potentials and that APP processing may be regulated by neuronal activity. The results from *in vivo* studies employing experimental cholinergic basal forebrain lesions have been inconsistent in regard to APP expression. While studies by Leanza, (1998b) and Lin et al., (1998) have reported a significant (up to 71%) increase in APP expression 6 months after i.c.v. 192-IgG-saporin injection in rats, Apelt et al., (1997) report no significant change in APP levels under similar conditions. This is an area that warrants further investigation. Furthermore, while it seems clear that cholinergic activity plays an important role in APP processing, there is little information available on how cholinergic activity might directly affect the activity or expression of certain proteins likely to mediate this effect, such as BACE, PS1, PS2 or TACE. Clearly, any modulation of APP processing by cholinergic basal forebrain activity will have implications relating to the formation of Aβ peptides. Despite the considerable amount of research that has described a loss of basal forebrain cholinergic neurons and also the accumulation of Aβ peptides in AD, it is not understood how these pathological changes may be related. While chapter 1 has addressed this issue in the context of how increased Aβ levels may affect cerebral cholinergic function, one of the aims of this chapter has been, conversely, to investigate how cholinergic basal forebrain activity affects the expression of APP and various proteins required for its processing and subsequent Aβ formation.

In summary, the aim of the work presented in this chapter has been to scrutinise the importance of a selective degeneration of basal forebrain cholinergic neurotransmission to the hippocampus and cortex in relation to the expression of a number of neurochemical markers known to be affected in these regions in AD. These neurochemical markers

including, nAChRs, NMDARs, nNOS, APP695, Presenilin 1 and 2, BACE and TACE were selected based on their relevance to human AD in terms of both neuropathology and potential as therapeutic targets. Considering the progressive nature of neurodegeneration in AD, it may be worthwhile to examine the effects of particular neuropathological changes over extended periods of time. In this context, the effect of cholinergic basal forebrain denervation on a temporal level was examined by quantifying these markers at a number of time points after i.c.v. injection of 192-IgG-saporin including 4, 7, 14, 30 and 90 days.

4.2 METHODS

4.2.1 TREATMENT OF ANIMALS AND TISSUE PREPARATION

Sprague-Dawley rats (250 – 300 g) of either sex were anaesthetised with pentobarbitone (60 mg/kg i.p.) and constrained to a tilted skull position (-0.3 mm) using a Kopf stereotaxic apparatus. A small hole was drilled into the skull and a stainless steel 30 gauge blunt tipped injection cannula was inserted into the left lateral ventricle at the following stereotaxic coordinates: 1.00 mm caudal to bregma, 1.50 mm left from the midline, 3.40 mm ventral from the cortical surface according to the atlas of Paxinos and Watson (1986). Injections of either 4 μg of 192-IgG-saporin (Chemicon Inc., USA) dissolved in 10 μl of PBS or 10 μl of PBS alone were delivered at the rate of approximately 2 μl/min. The cannula was left in place for a further 10 min following injection to allow adequate diffusion of the injected contents from the injection site. Post injection recovery periods of 4, 7, 14, 30 and 90 days were chosen for analysis. The experimental paradigm therefore consisted of 10 groups including: 4, 7, 14, 30 and 90 day sham-operated (vehicle injected) and 4, 7, 14, 30 and 90 day 192-IgG-saporin treated. Each group consisted of 4 rats. At the end of the post-operative recovery periods, the animals were decapitated and the brains frozen over liquid nitrogen for storage at -70°C.

For histochemical and autoradiographic analysis, a Reichert Jung cryostat was used to collect a series of 14 μm sections. Consecutive coronal sections were taken at 3.6 mm caudal to bregma and thaw mounted on poly-L-lysine coated slides. All tissue was stored at -70°C until required.

4.2.2 AChE HISTOCHEMISTRY

AChE levels were quantified using a modification of the method previously described by Koelle et al., (1949). This method has been described in detail under Chapter 2, General Methodology, *2.4 AChE histochemistry.*

4.2.3 IN SITU HYBRIDISATION HISTOCHEMISTRY

The protocol for in situ hybridisation is based on the methods described by Wisden et al., (1994) and adapted by Loiacono et al., (1999), and has been described in detail under Chapter 2, General Methodology, *2.1 In Situ Hybridisation Histochemistry.*

4.2.4 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY

The protocol for [^{3}H]epibatidine autoradiography was based on that described by Perry et al., (1995b) and has been described in detail under Chapter 2, General Methodology, *2.2 [^{3}H]Epibatidine Autoradiography.*

4.2.5 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY

The protocol for [^{125}I]α-BGT was based on that used by Whiteaker et al., (1999) and has been described in detail under Chapter 2, General Methodology, *2.3 [^{125}I]α-Bungarotoxin Autoradiography.*

4.2.6 QUANTITATION

Images generated through the *in situ* hybridisation histochemistry, histochemical and binding protocols were digitally captured and selected anatomical regions were quantified using Scion imaging software. For mRNA images, [^{125}I]α-BGT autoradiographs and AChE histochemical stains the system was calibrated against a set of graded greyscales (Kodak), allowing for quantitation in terms of relative optical density (ROD). For [^{3}H]epibatidine autoradiographs, the system was calibrated against a set of standard tritium scales, allowing for quantitation in terms of nCi/mm^2. Differences in ROD or nCi/mm^2 between 192-IgG-saporin and vehicle treated groups were assessed statistically using either an unpaired student's t test or one-way ANOVA with a Dunnett post test depending on the number of groups being analysed. Statistical significance was signified by P values of less then 0.5. Values reported here represent the mean of measurements taken from 4 animals. Issues relating to quantitation have been described in greater detail in Chapter 2, General Methodology, *2.5 Quantitation.*

4.3 RESULTS

4.3.1 AChE HISTOCHEMISTRY

AChE levels were quantified through densitometric analysis in the dentate gyrus, Ca1, Ca2 and Ca3 fields of the hippocampus, cortical layers 1 – 3 and 4 – 6, medial septum, caudate putamen, as well as amygdaloid and thalamic nuclei as defined in the atlas of Paxinos and Watson (1986). A number of heterogenous groups of thalamic nuclei were included in the quantification of AChE levels in the thalamus, including primarily mediodorsal thalamic nuclei. Representative AChE stained sections from each treatment group are shown in figure 4.2 (telencephic areas) and figure 4.3 (caudate putamen and medial septum). The level of AChE staining was visibly reduced throughout the hippocampus and cortex in 192-IgG-saporin treated rats relative to vehicle treated rats. At 4 and 7 days post-treatment there was clearly a bilateral difference with regard to this effect, with the greatest reduction in AChE levels occurring in the 192-IgG-saporin injected (left) hemisphere. However, for the purposes of this study, measurements were always taken from the left hemisphere. No significant difference was seen in the level of AChE between the 4, 7, 14, 30 and 90-day vehicle infused groups (data not shown). Therefore, measurements taken from the 14-day post-operative vehicle treated group were used to represent AChE levels in vehicle treated animals for comparison with AChE levels in the 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups.

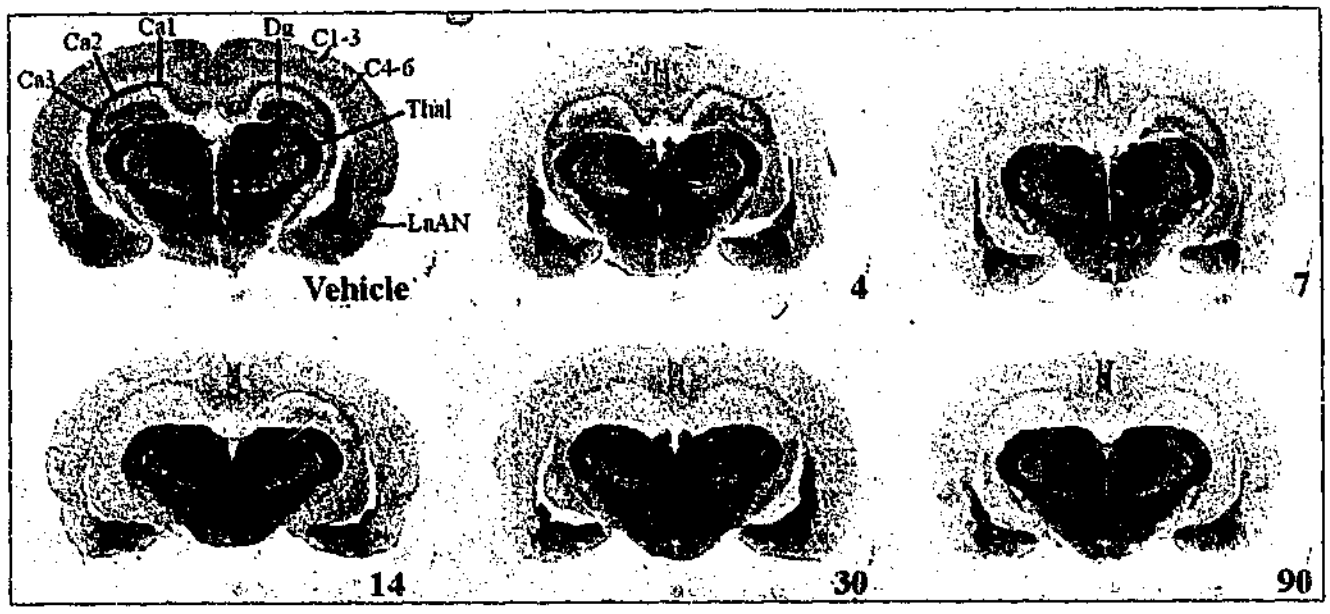


Figure 4.2. AChE histochemistry in representative coronal sections approximately 3.6 mm caudal to bregma from vehicle treated and 4, 7, 14, 30 and 90-day post-operative 192-IgG-saporin treated groups. Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3, C4-6, cortical layers 4-6; Dg, dentate gyrus; LaAN, lateral amygdaloid nuclei; Thal, thalamic nuclei. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

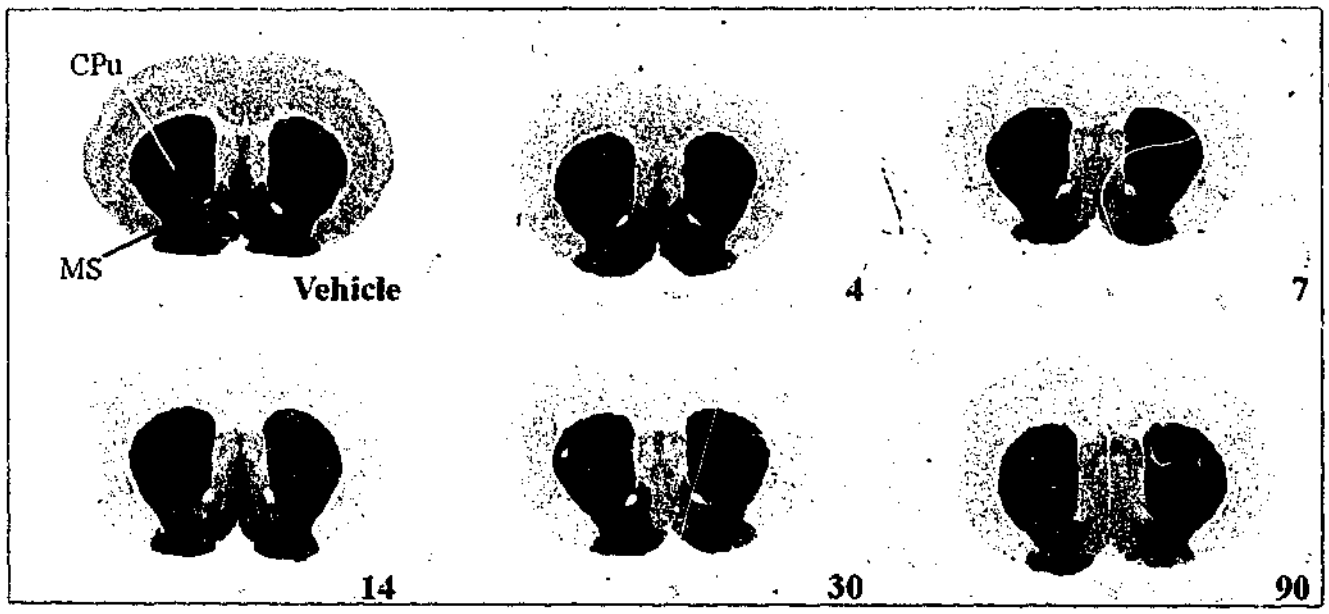


Figure 4.3. AChE histochemistry in representative coronal sections approximately 0.70 mm rostral to bregma from vehicle treated and 4, 7, 14, 30 and 90-day post-operative 192-IgG-saporin treated groups. Abbreviations: CPu, caudate putamen; MS, medial septum.

Rats treated with 192-IgG-saporin exhibited a significant loss in AChE staining in the hippocampus and cortex, the degree of which varied depending on the post-operative time point examined (fig 4.4). In the hippocampal regions, there was a progressive loss which peaked at the 7 – 14 day post-operative time points, after which time there seemed to be some recovery of AChE levels in the 30 and 90 day post-operative groups. The

AChE loss in the cortex was not as progressive as that seen in the hippocampus with the greatest loss seen at the earliest, 4-day post-operative time point. After the 4-day time point, there was a progressive recovery of cortical AChE levels and this was more prominent then was seen in the hippocampus. There was also a significant and progressive loss in AChE levels in the medial septum at 7, 14, 30 and 90 days post-treatment (fig. 4.5). This effect was greatest at the 14 day time point and persisted at the 30 and 90 day time points with no indication of a recovery of AChE levels as was seen in the hippocampus and cortex. There was no significant change in AChE levels in the caudate putamen or thalamic or amagdaloid nuclei analysed, at any of the time points after 192-IgG-saporin treatement.

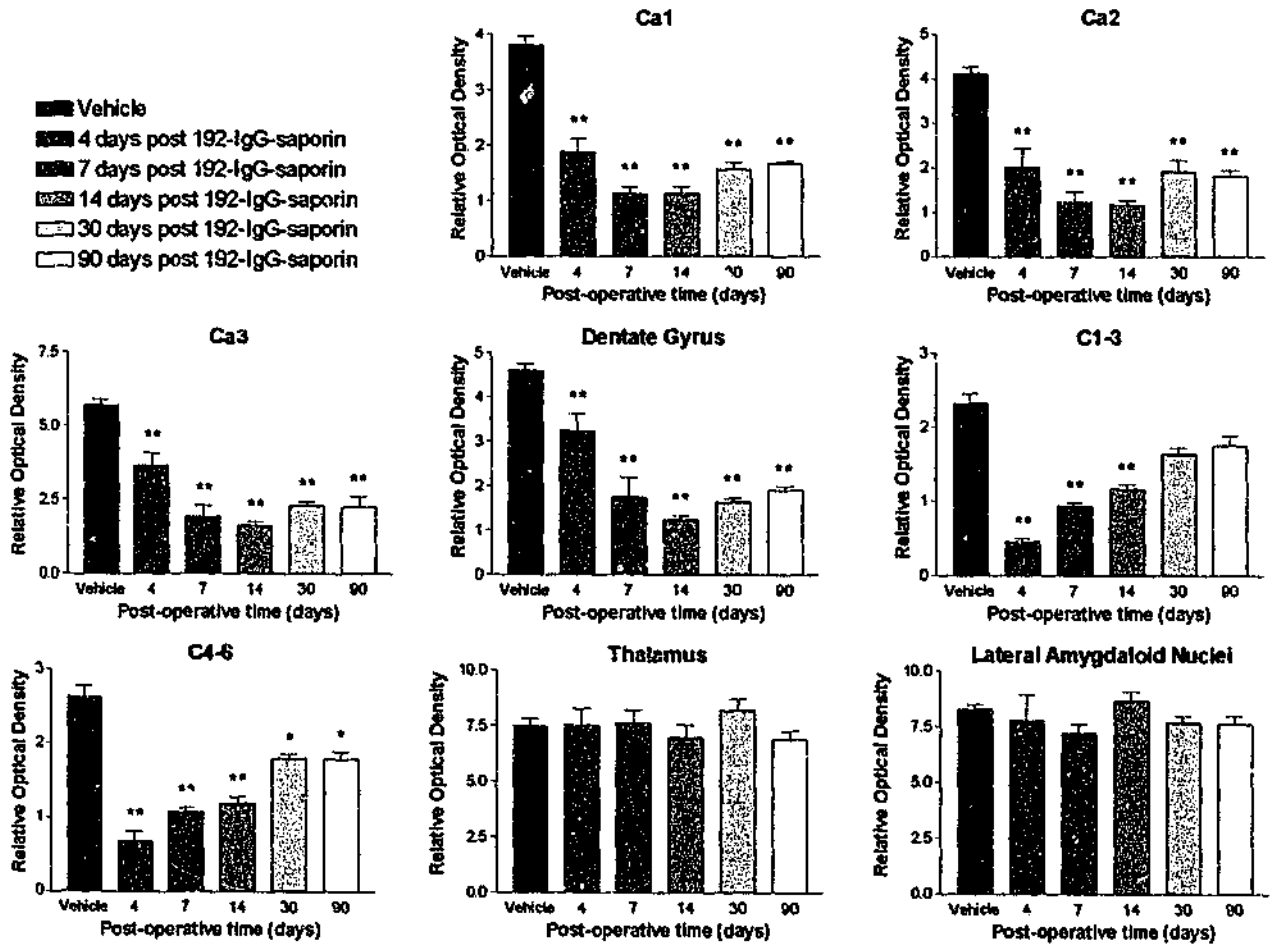


Figure 3.3. AChE levels in selected telencephic regions in coronal sections approximately 3.6 mm caudal to bregma from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4-6, cortical layers 4-6. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using a one-way ANOVA with a Dunnett post-test (*, $P < 0.05$; **, $P < 0.01$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

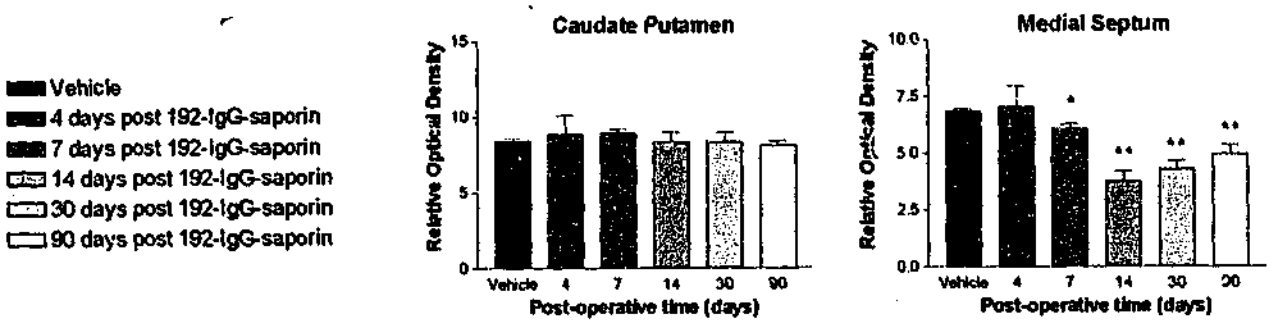


Figure 4.5. AChE levels in the caudate putamen and medial septum from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using a one-way ANOVA with a Dunnett post-test (*, $P < 0.05$; **, $P < 0.01$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

4.3.2 nAChR SUBUNIT mRNA EXPRESSION

The level of nAChR subunit mRNA expression was examined by *in situ* hybridisation histochemistry in coronal sections from rats in each treatment group. Figure 4.6 shows the unique anatomical distribution of α_4, α_7 and β_2 nAChR subunit mRNA expression in various telencephic regions of the rat brain.

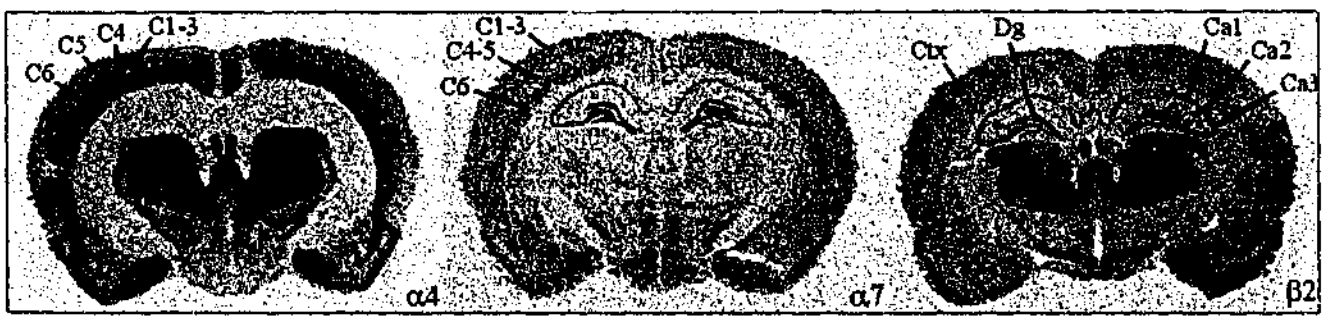


Figure 4.6. Distribution of nAChR α_4, α_7, and β_2 mRNA subunit expression in representative coronal sections approximately 3.6 mm caudal to bregma. Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4, cortical layer 4; C4-5, cortical layers 4-5; C5, cortical layer 5; C6, cortical layer 6; Ctx, cortex; Dg, dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Generally, the levels of expression of the α_4, α_7 and β_2 nAChR subunits remained relatively stable after 192-IgG-saporin treatment at all time points in the regions examined in this study (fig.'s 4.7 – 4.9). At the 4 and 7 day post-operative time points, there was a

small increase of 6 – 32% and 10 – 33% respectively in the expression of α_4 subunit mRNA throughout the cortex in the 192-IgG-saporin treated group relative to vehicle treated controls. This was not a statistically significant result however. At the 14-day time point there was a moderate *decrease* of 16 – 31% in α_4 expression in the cortical layers of the 192-IgG-saporin group. This was a statistically significant result in cortical layer 6. At the 30 and 90 day time points there was a significant decrease in α_7 subunit mRNA expression in 192-IgG-saporin treated rats in the Ca3 area of the hippocampus and the dentate gyrus respectively.

Overall, as a result of 192-IgG-saporin treatment there were some small changes in the level of α_4 subunit mRNA expression at early time points (4, 7 and 14 days; fig. 4.7) and α_7 mRNA expression at later time points (30 and 90 days; fig 4.9), while the level of β_2 expression was not affected at any of the time points examined (fig 4.8).

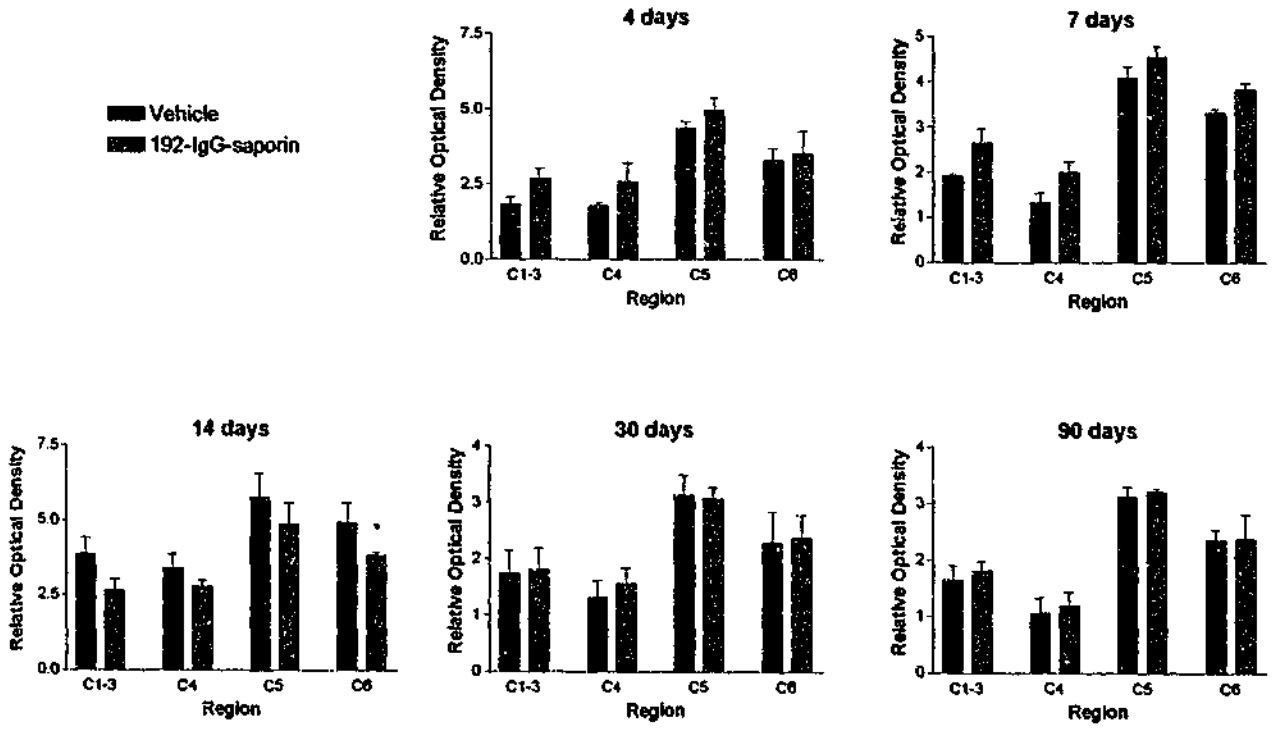


Figure 4.7. Levels of α_4 nAChR subunit mRNA in selected cortical layers from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: C1-3, cortical layers 1-3; C4, cortical layer 4; C5, cortical layer 5; C5, cortical layer 5; C6, cortical layer 6. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test (*, $P < 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

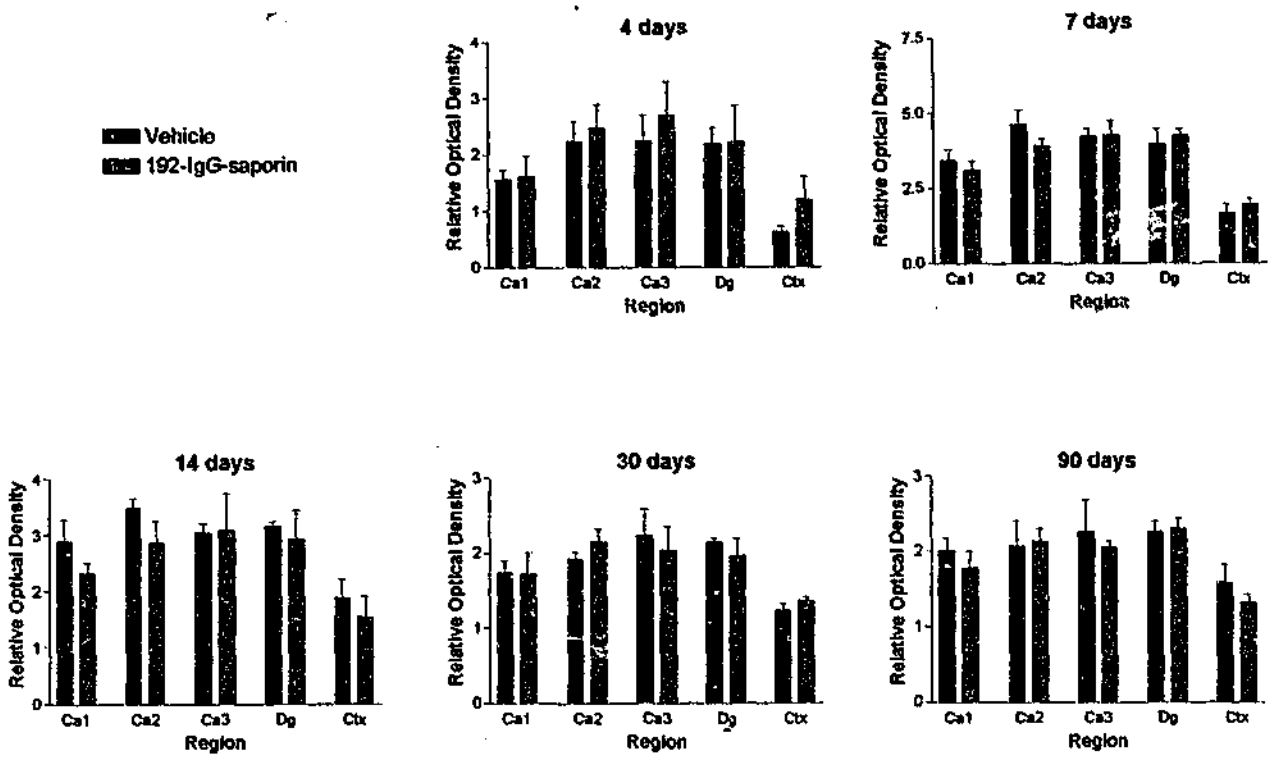


Figure 4.8. Levels of β_2 nAChR subunit mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. The mean ROD in the 192-IgG-saporin treated group was compared to the mean level of ROD in the vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

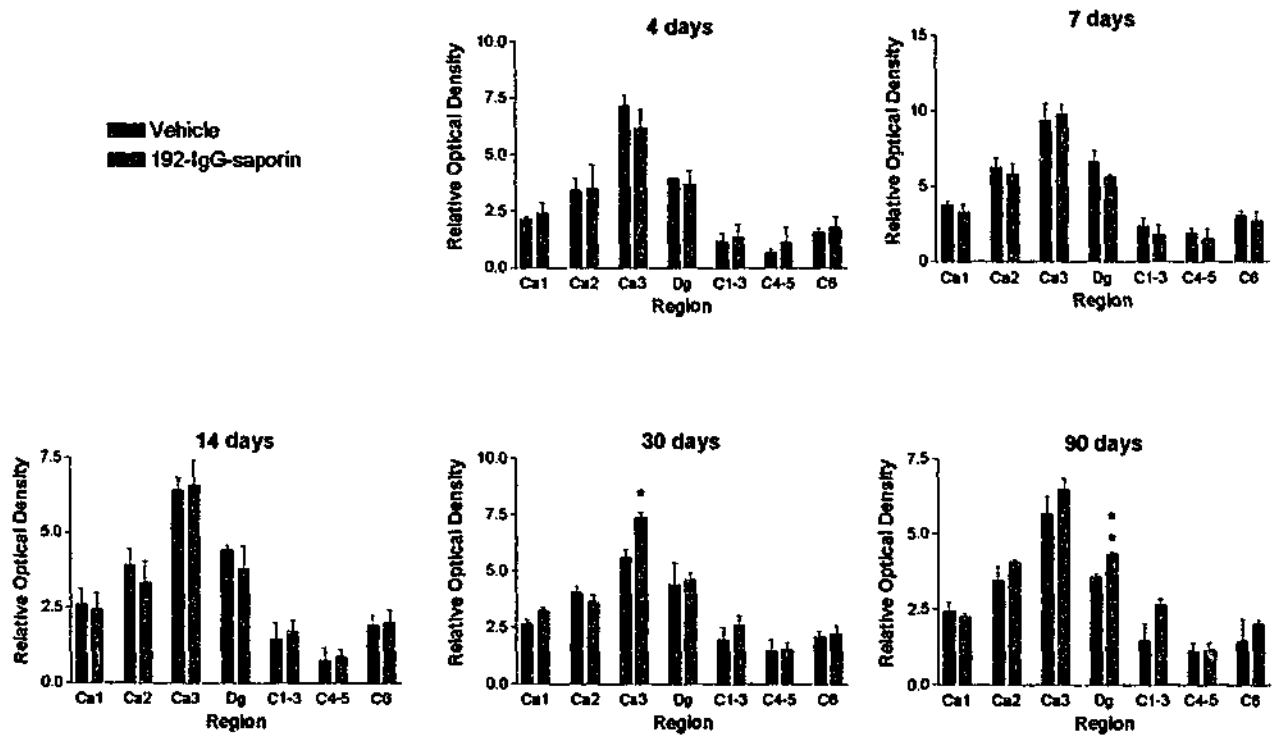


Figure 4.9. Levels of α_7 nAChR subunit mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4-5, cortical layers 4-5; C6, cortical layer 6. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test (*, $P < 0.05$; **, $P < 0.01$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

4.3.3 [^{3}H]Epibatidine Autoradiography

Nicotinic receptor levels were assessed in rats from each treatment group by [^{3}H]epibatidine autoradiography. Epibatidine binds predominately to nAChRs of the $\alpha_4\beta_2$ subtype and also, to a lesser extent, to other subtypes such as the $\alpha_3\beta_2$ receptor, which are expressed at much lower levels. The regional distributuion of [^{3}H]epibatidine binding in a representative section is shown in figure 4.10. Non-specific binding, as defined by 300 μM (−)-nicotine, was undetectable in all cases (data not shown).

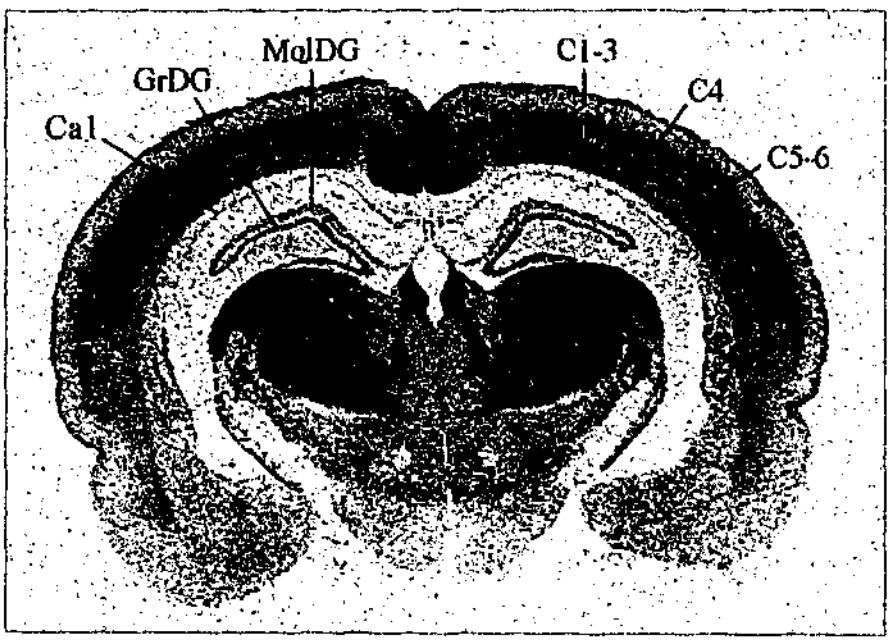


Figure 4.10. Total [^{3}H]Epibatidine binding in a representative coronal section approximately 3.6 mm caudal to bregma. Abbreviations: Ca1, field of the hippocampus; C1-3, cortical layers 1-3; C4, cortical layer 4; C5-6, cortical layers 5-6; GrDG, granular layer dentate gyrus; MolDG, molecular layer dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Rats treated with 192-IgG-saporin did not show any significant change in the level [^{3}H]epibatidine binding, relative to vehicle injected animals, in any of the regions examined in this study (unpaired student's t test, $P > 0.05$; fig 4.11).

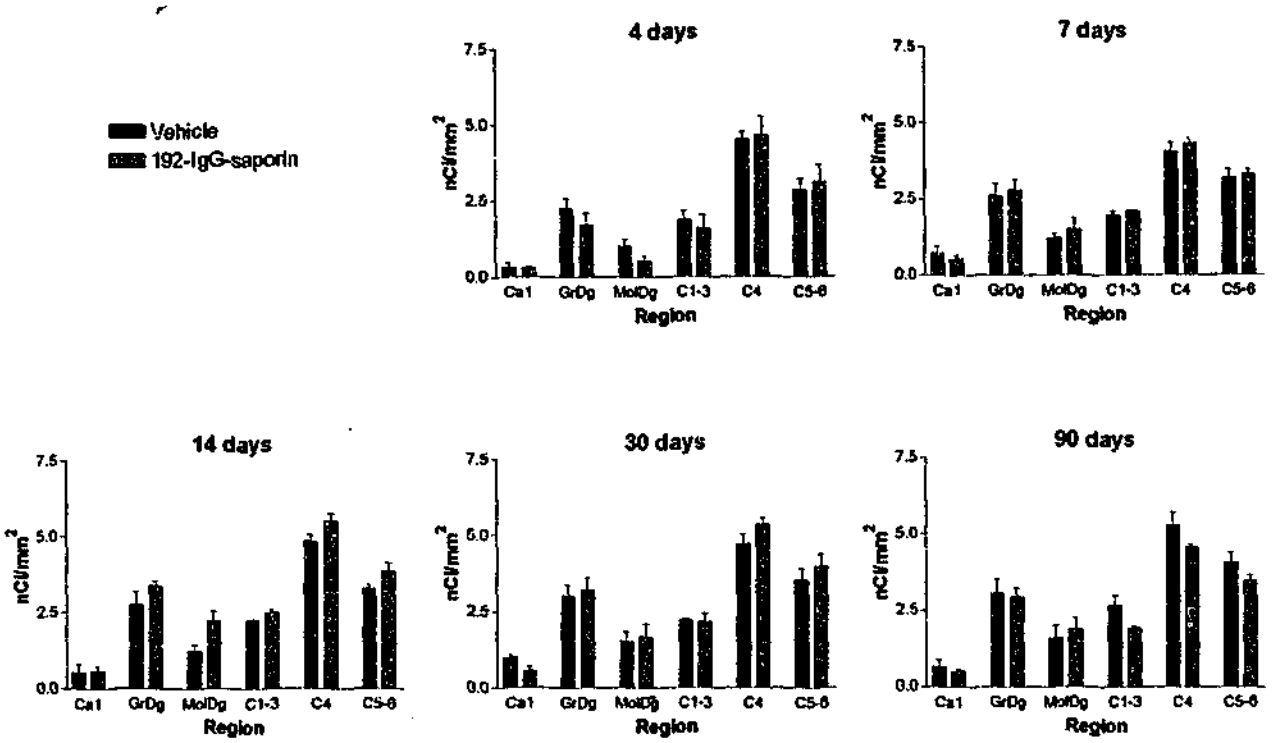


Figure 4.11. Specific [^{3}H]Epibatidine binding in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of nCi/mm^2. Abbreviations: Ca1, field of the hippocampus; C1-3, cortical layers 1-3; C4, cortical layer 4; C5-6, cortical layers 5-6; GrDG, granular layer dentate gyrus; MolDG, moleculer layer dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean nCi/mm^2 level in the 192-IgG-saporin treated groups was compared to the mean level of nCi/mm^2 in the corresponding vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

4.3.4 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY

The density of α_7 nAChRs in vehicle and saporin injected rats was assessed in the Ca1, Ca2, and Ca3 fields of the hippocampus, the hippocampal fissure, the stratum radiatum area of the hippocampus and cortical layers 1-5 and 6 as defined in the atlas of Paxinos and Watson (1986) by autoradiography of the α_7 nAChR selective ligand [^{125}I]α-BGT. The regional distribution of [^{125}I]α-BGT binding to α_7 nAChRs in a representative coronal section is shown in figure 4.12. Non-specific binding, as defined by 10 mM (–)-nicotine, ranged from <10% in the dentate gyrus to approximately 40% in the stratum radiatum hippocampus (data not shown) and was subtracted from the total signal during quantitation.

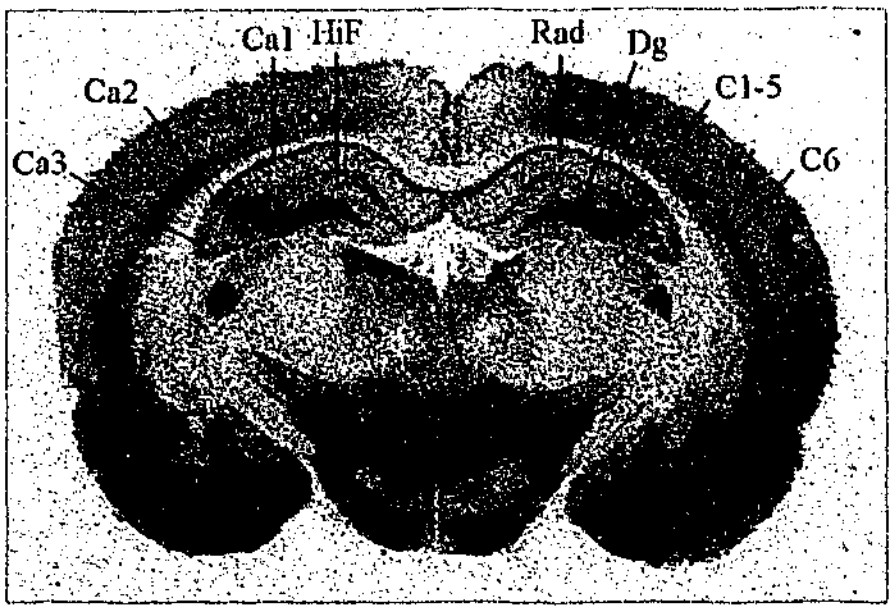


Figure 4.12. Total [^{125}I]α-BGT binding in a representative section approximately 3.6mm caudal to bregma. Abbreviations: Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

[^{125}I]α-BGT binding was seen throughout the hippocampus and cortex, with the signal being particularly strong in the dentate gyrus. There was no significant change in the level of [^{125}I]α-BGT binding as a result of 192-IgG-saporin treatment relative to vehicle injected animals at any of the time points examined in any of the regions quantified (unpaired student's t test, $P > 0.05$; fig. 4.13).

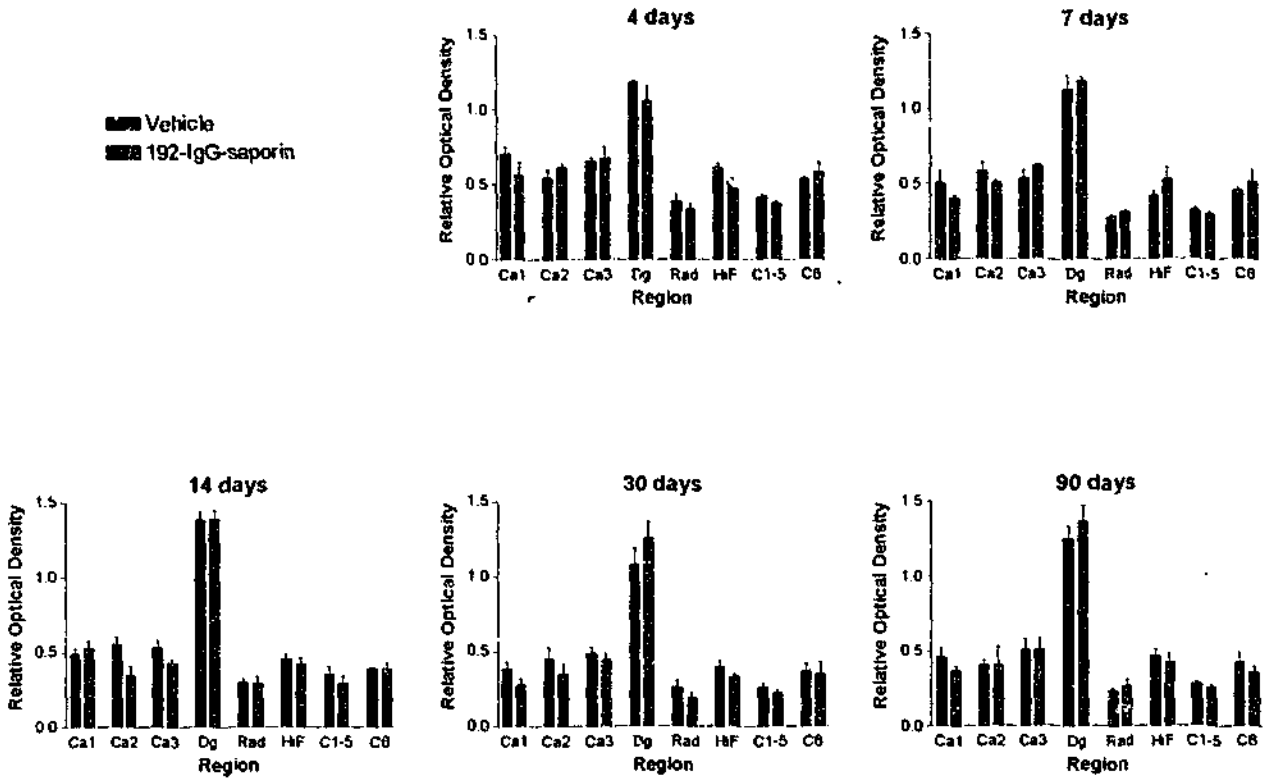


Figure 4.13. Specific [^{125}I]α-BGT binding in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

4.3.5 NMDA Receptor Subunit mRNA Expression

NMDAR NR1, NR2A and NR2B subunit mRNA expression was examined by *in situ* hybridisation histochemistry in coronal sections from vehicle and 192-IgG-saporin treated rats at 4, 7, 14, 30 and 90 days after treatment. Representative autoradiographs showing the density and localisation of NR1, NR2A and NR2B NMDAR subunit mRNA expression are shown in figure 4.14.

Figure 4.14. NMDAR NR1, NR2A and NR2B subunit mRNA expression in representative coronal sections taken approximately 3.6 mm caudal to bregma. Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex.

Deafferentation of the cholinergic basal forebrain system following 192-IgG-saporin treatment had relatively little effect on the level of expression of the NR1 (fig. 4.15), NR2A, (fig.'s 4.16 and 4.17) or NR2B (fig. 4.18) subunit mRNAs, relative to vehicle treated controls. At the 90 day post-operative time point, however, there was a modest increase of 8 – 15%, 8 – 40% and 14 – 35% in the level of expression of mRNA for the NR1, NR2A and NR2B subunits respectively in the various regions examined of 192-IgG-saporin treated rats relative to the vehicle treated rats. This increase was only statistically significant with regard to NR2A expression in the Ca1 field of the hippocampus and the cortex.

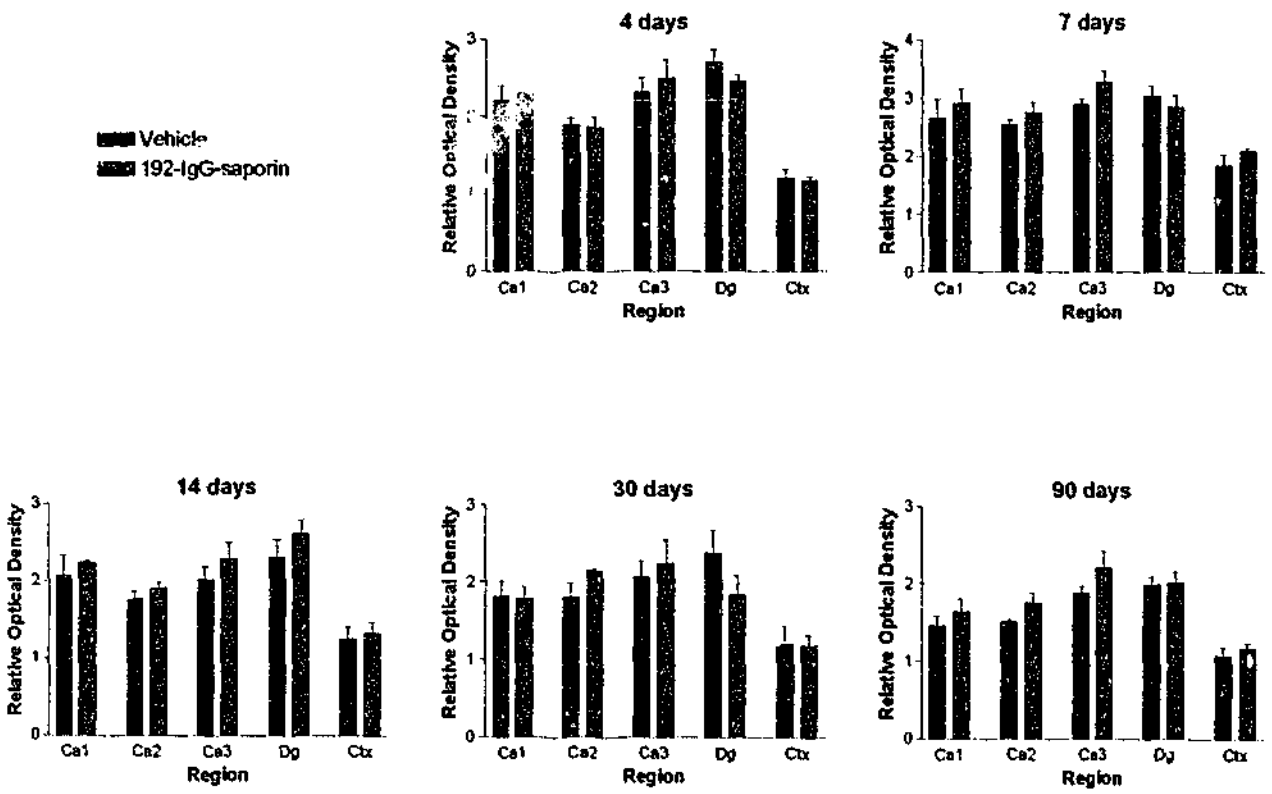


Figure 4.15. Levels of NR1 NMDAR subunit mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

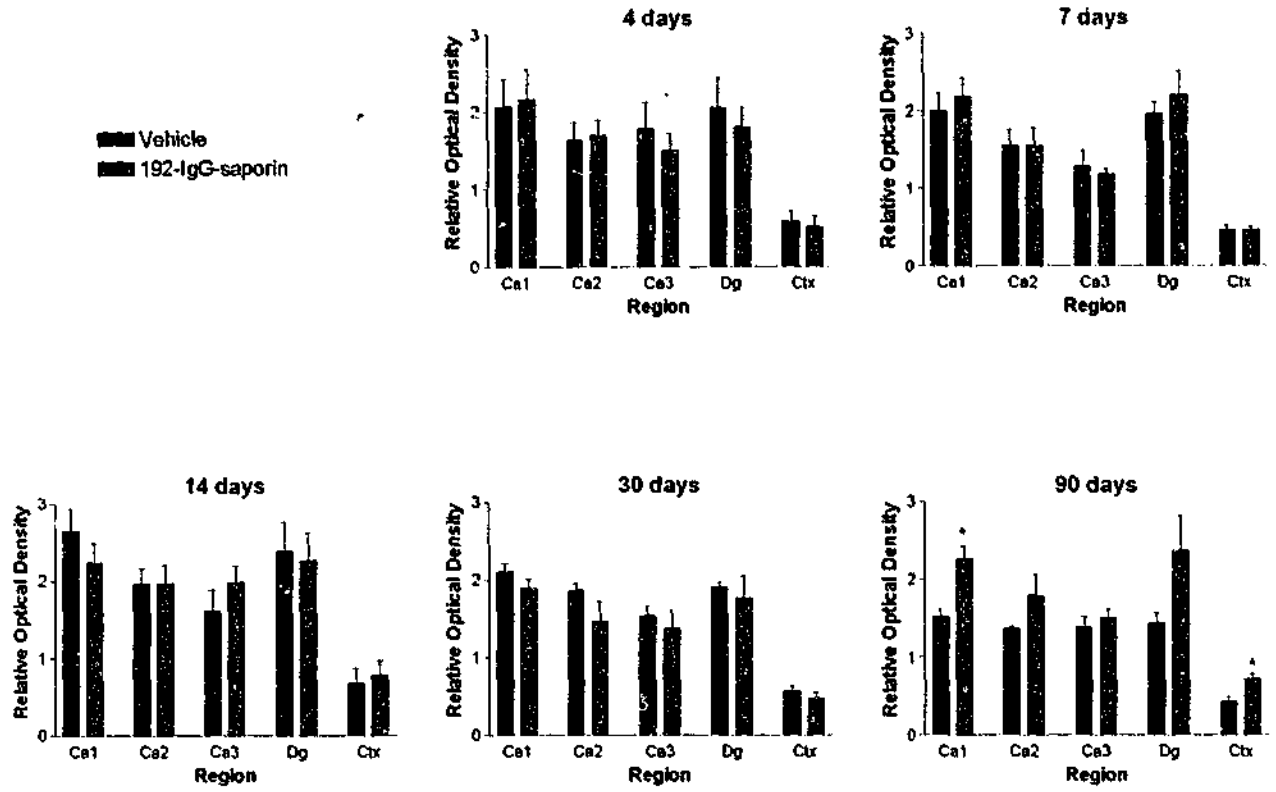


Figure 4.16. Levels of NR2A NMDAR subunit mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test (*, $P < 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

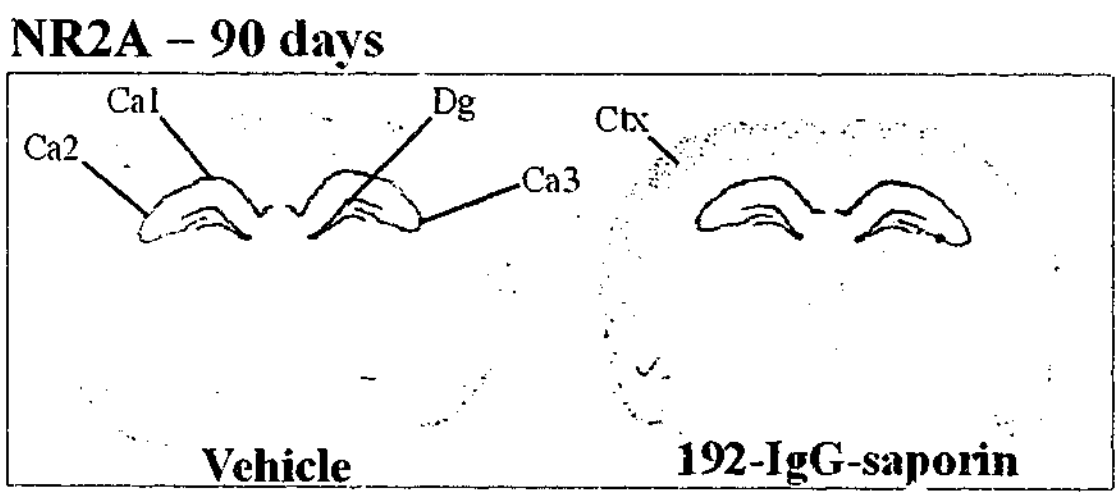


Figure 4.17. NR2A subunit mRNA expression in representative coronal sections taken approximately 3.6 mm caudal to bregma from vehicle and 192-IgG-saporin treated rats 90 days after surgery. Abbreviations: Ctx, cortex; Dg, dentate gyrus.

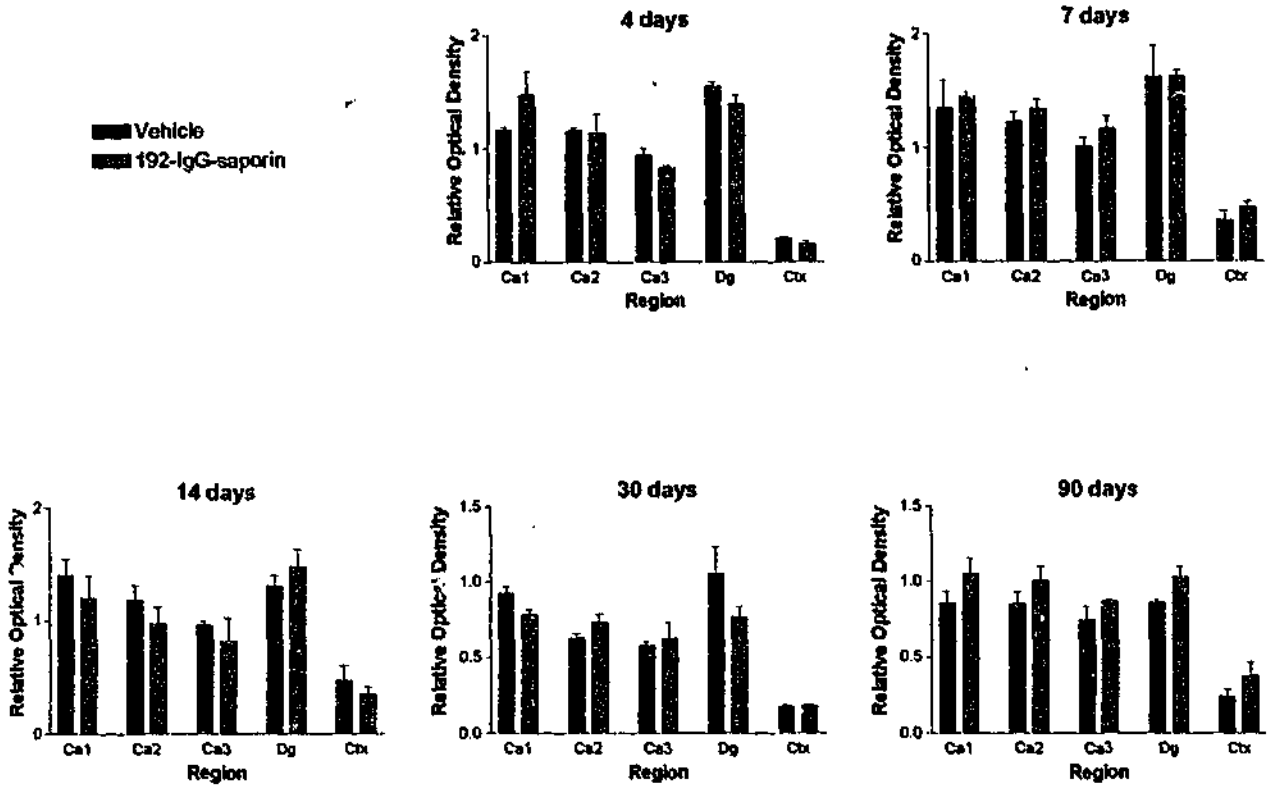


Figure 4.18. Levels of NR2B NMDAR subunit mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

4.3.6 nNOS mRNA EXPRESSION

The level of nNOS mRNA expression in rats treated with either vehicle or 192-IgG-saporin was assessed by *in situ* hybridisation histochemistry in the Ca1, Ca2, and Ca3 fields of the hippocampus, dentate gyrus and cortex. The distribution of nNOS mRNA expression following vehicle treatment and at the various time points after 192-IgG-saporin treatment is shown in figure 4.19.

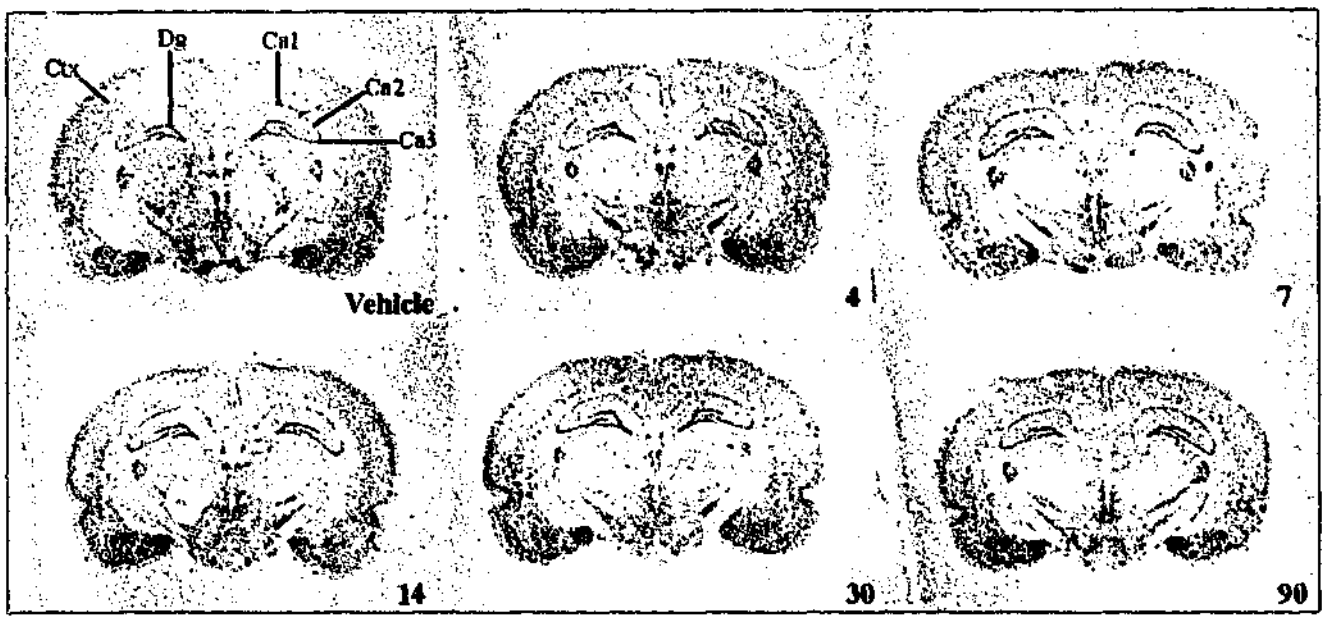


Figure 4.19. nNOS mRNA expression in representative coronal sections taken approximately 3.6 mm caudal to bregma from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups. Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus.

Rats treated with i.c.v. injections of 192-IgG-saporin exhibited a significant increase of 28 – 30% in the expression of nNOS mRNA in the dentate gyrus at 7, 14, 30 and 90 days after treatment (fig. 4.20). There was also an increase in expression of 15 – 36% in the Ca1 of the hippocampus over these time points, however this affect was only statistically significant at 14 days post-treatment. There was no significant difference in nNOS expression in any region 4 days after treatment although there appeared to be a small *reduction* in nNOS levels in the 192-IgG-saporin treated group throughout the hippocampus, relative to vehicle treated animals at this early timepoint.

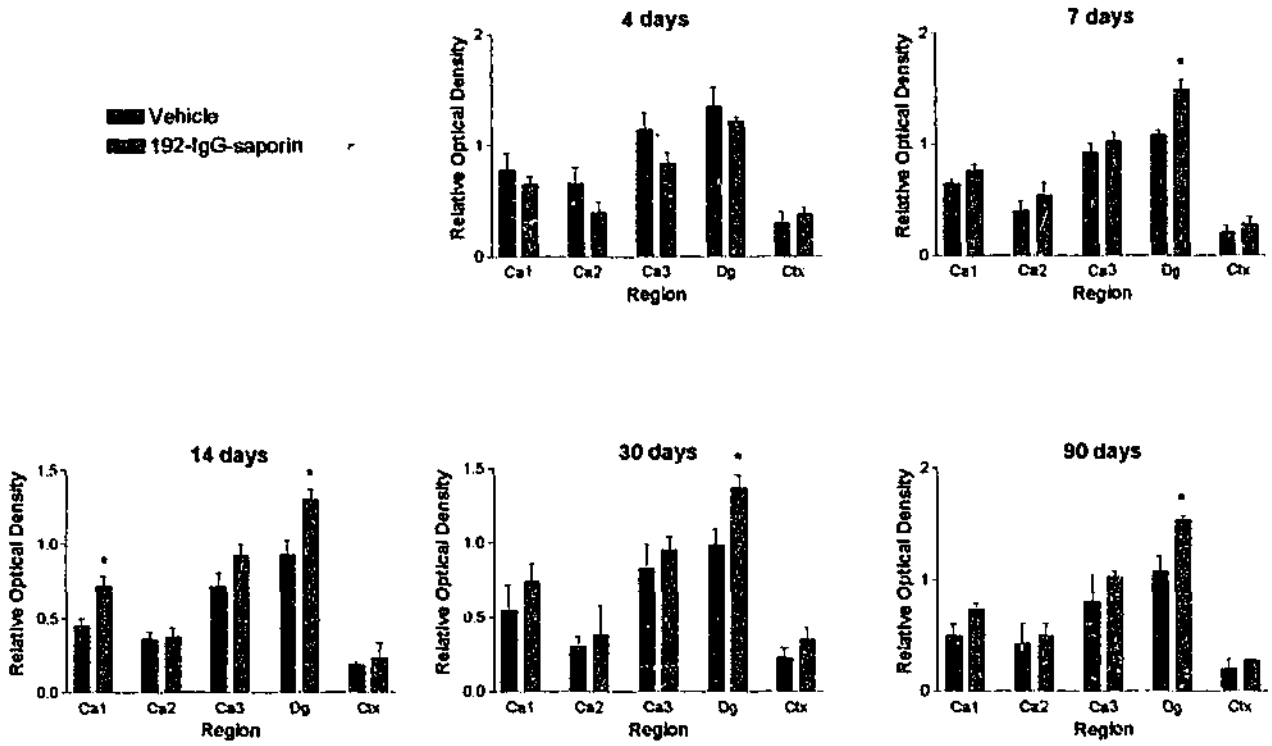


Figure 4.20. Levels of nNOS mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test (*, $P < 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

4.3.7 APP695, BACE, PS1, PS2 AND TACE mRNA EXPRESSION

The mRNA expression of APP695, BACE, PS1, PS2 and TACE in coronal sections from vehicle and 192-IgG-saporin treated rats was examined using *in situ* hybridisation histochemistry. Treatment with 192-IgG-saporin resulted in an increase in the level of APP695 mRNA expression, which was not evident until 90 days after the initial treatment. The distribution of APP695 mRNA in representative coronal sections from each treatment group is shown below in figure 4.21.

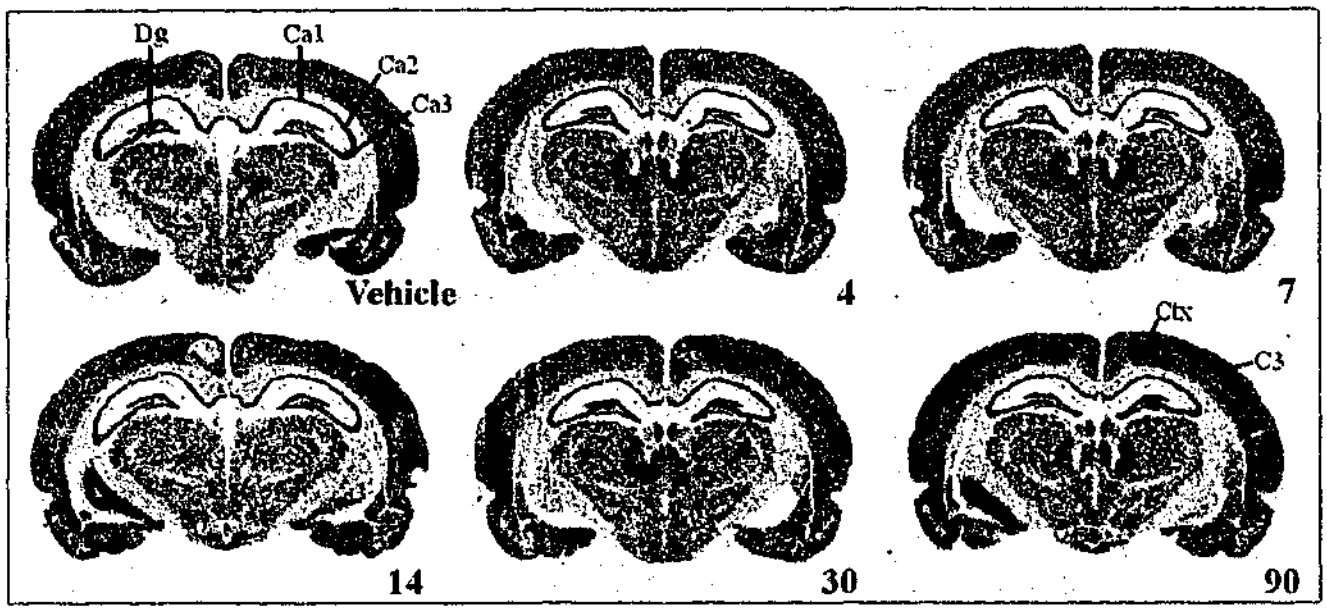


Figure 4.21. Distribution of APP695 mRNA in representative coronal sections approximately 3.6 mm caudal to bregma from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated rats. Abbreviations: Ca1-3, fields of the hippocampus; C3, cortical layer 3; ctx, cortex; Dg, dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Densiometric analysis of the coronal images revealed that, relative to i.c.v. injection of vehicle, there was a statistically significant increase in APP695 mRNA levels in the cortex of rats injected with 192-IgG-saporin at 90 days after treatment, but not at the earlier time points examined (fig. 4.22).

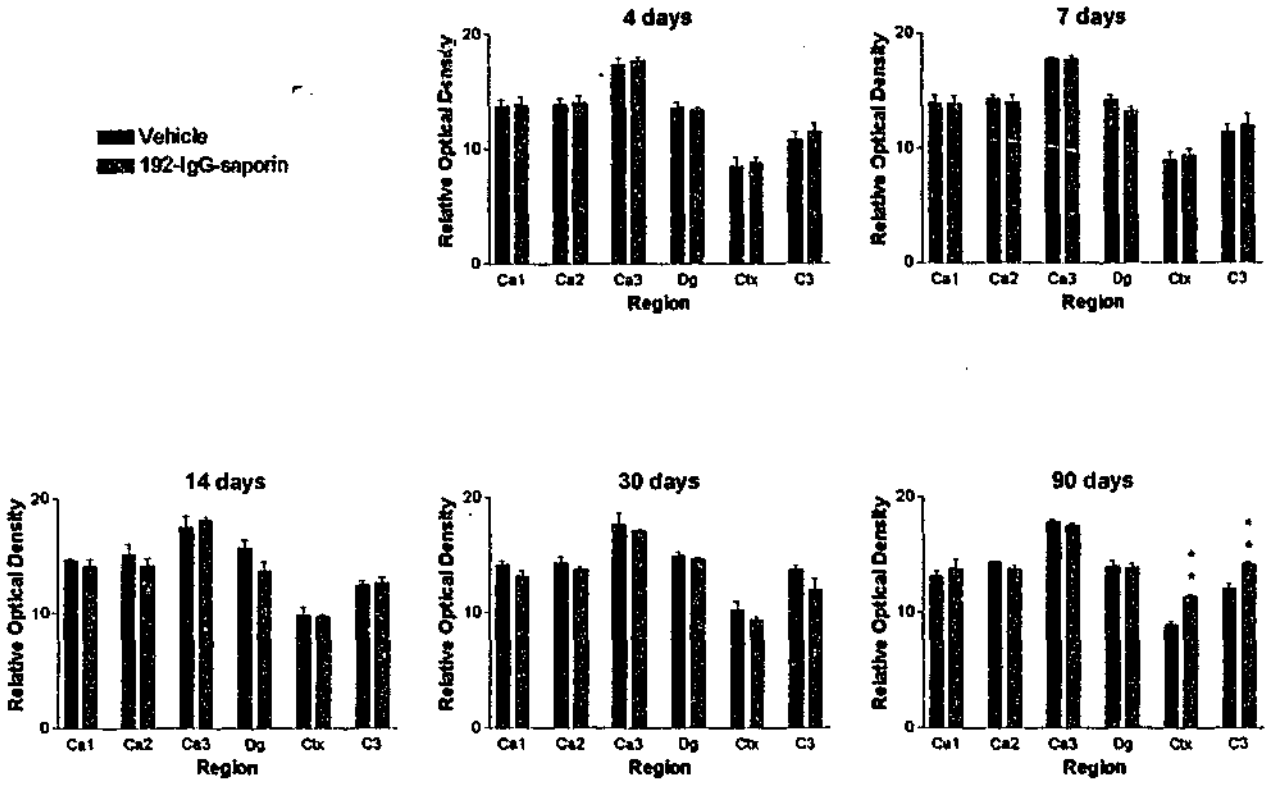


Figure 4.22. Levels of APP695 mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; C3, cortical layer 3; Dg, dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test (**, $P < 0.01$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

The density and localisation of BACE, PS1, PS2 and TACE mRNA in representative coronal sections from vehicle treated rats is shown in figure 4.22.

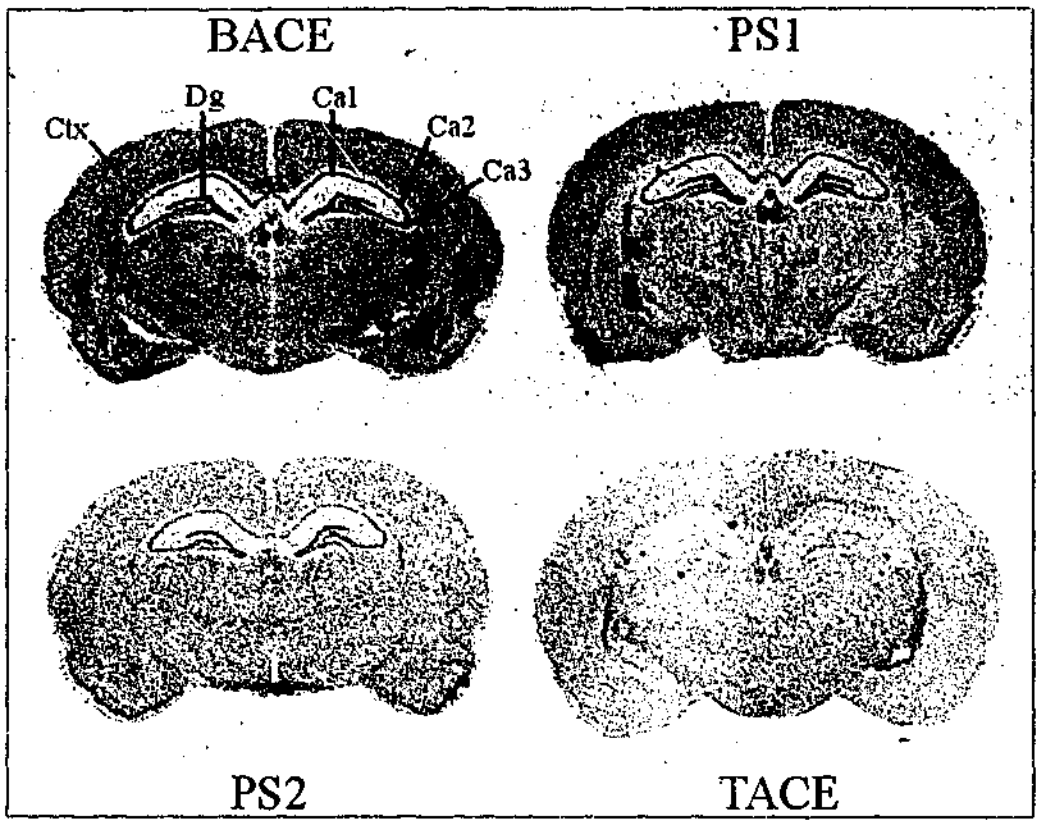


Figure 4.23. Distribution of β-site APP-cleaving enzyme (BACE), presenilin-1 (PS1), presenilin-2 (PS2) and tumour necrosis factor-α-converting enzyme (TACE) mRNA in representative coronal sections approximately 3.6 mm caudal to bregma from vehicle treated rats. Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

No significant change in the level of BACE, PS1, PS2 or TACE mRNA expression was seen in the hippocampus or cortex of rats treated with 192-IgG-saporin at any of the post-operative time points examined, relative to vehicle treated animals ($p > 0.05$, unpaired student's t test; fig.'s. 4.24 – 4.27).

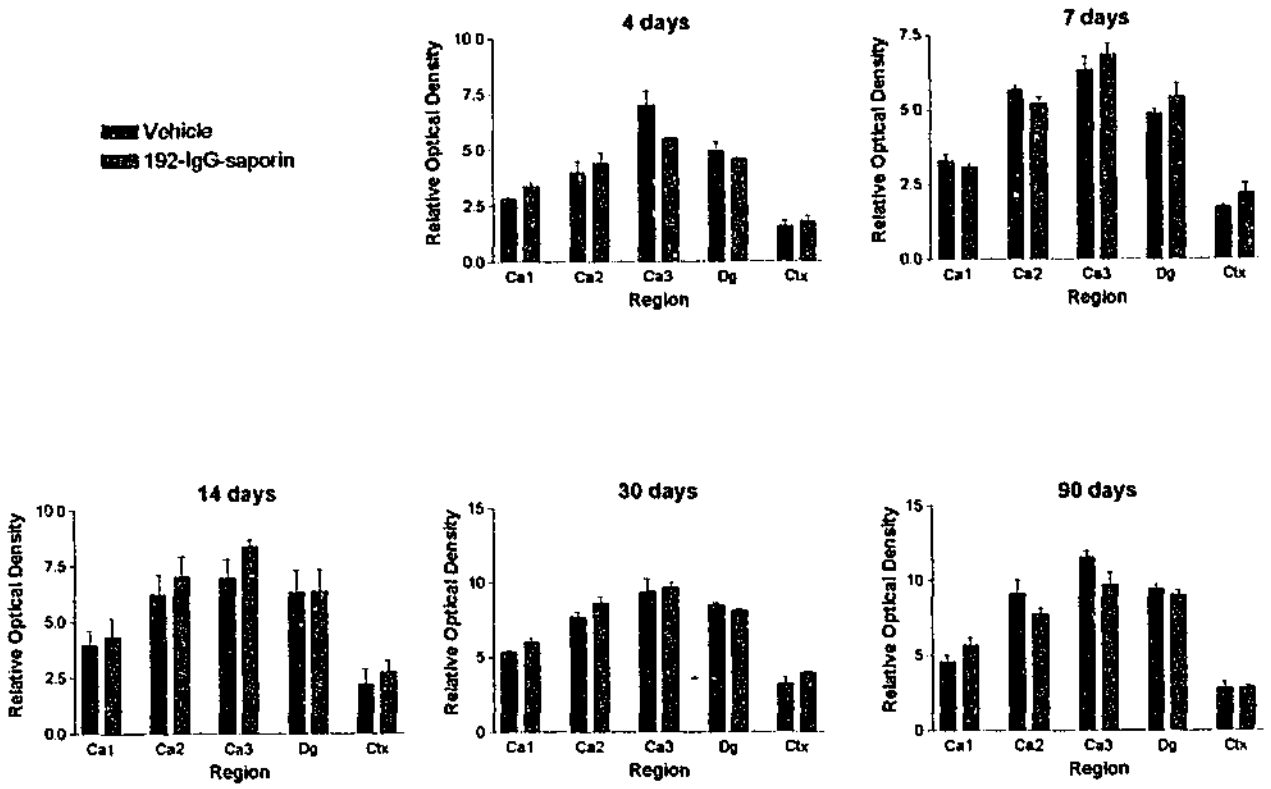


Figure 4.24. Levels of β-site APP-cleaving enzyme (BACE) mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

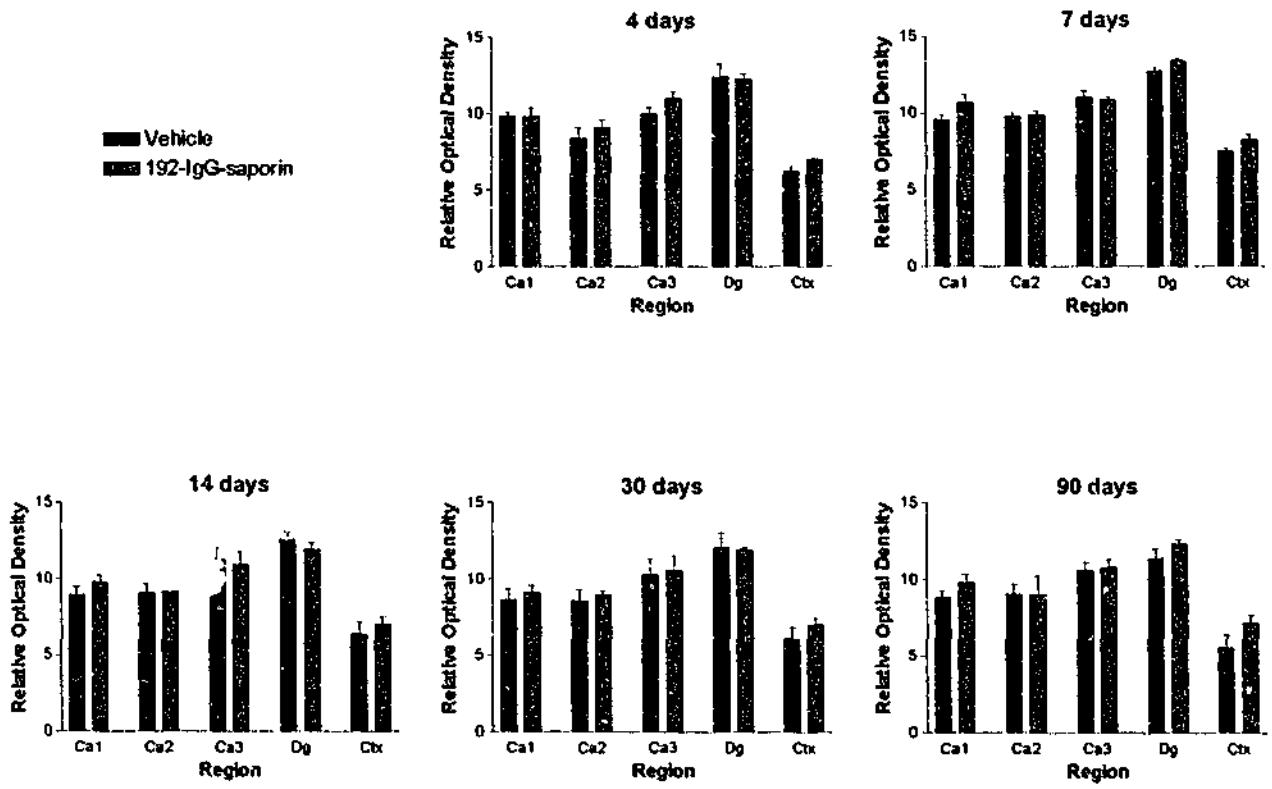


Figure 4.25. Levels of presenilin-1 (PS1) mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

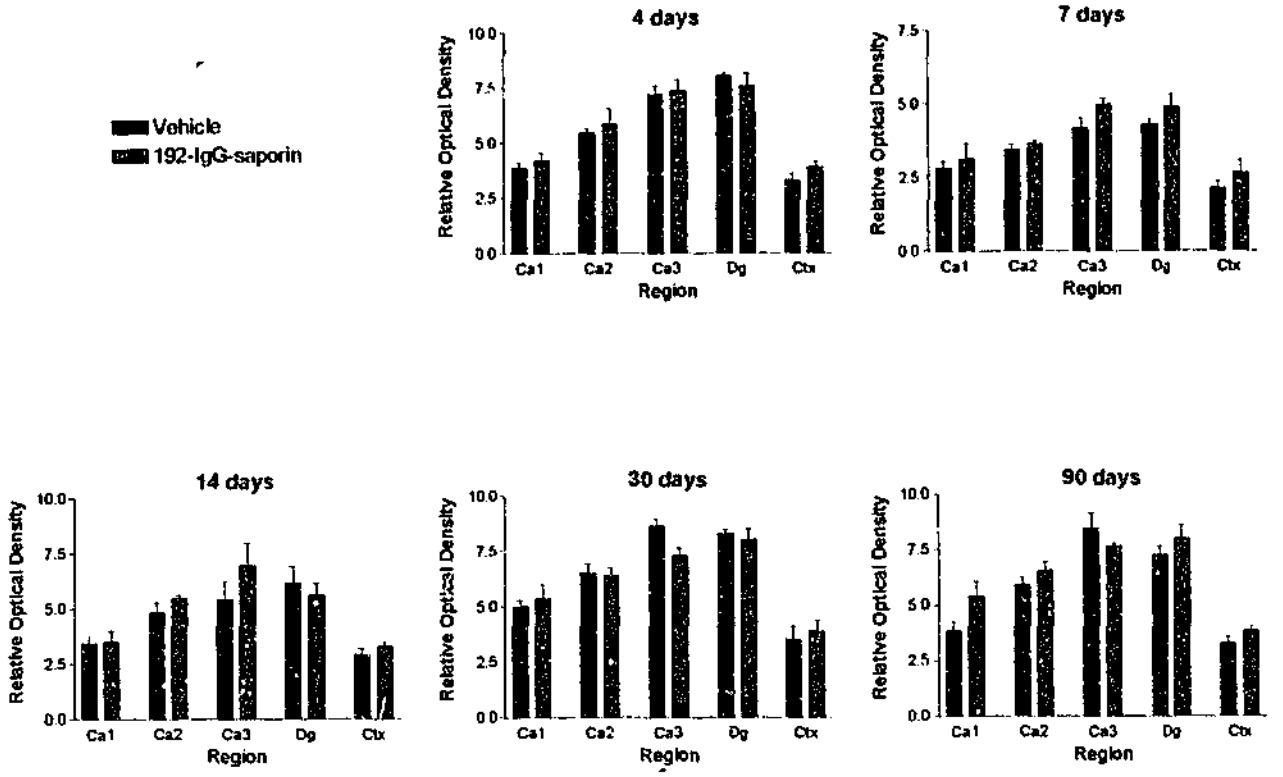


Figure 4.26. Levels of presenilin-2 (PS2) mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

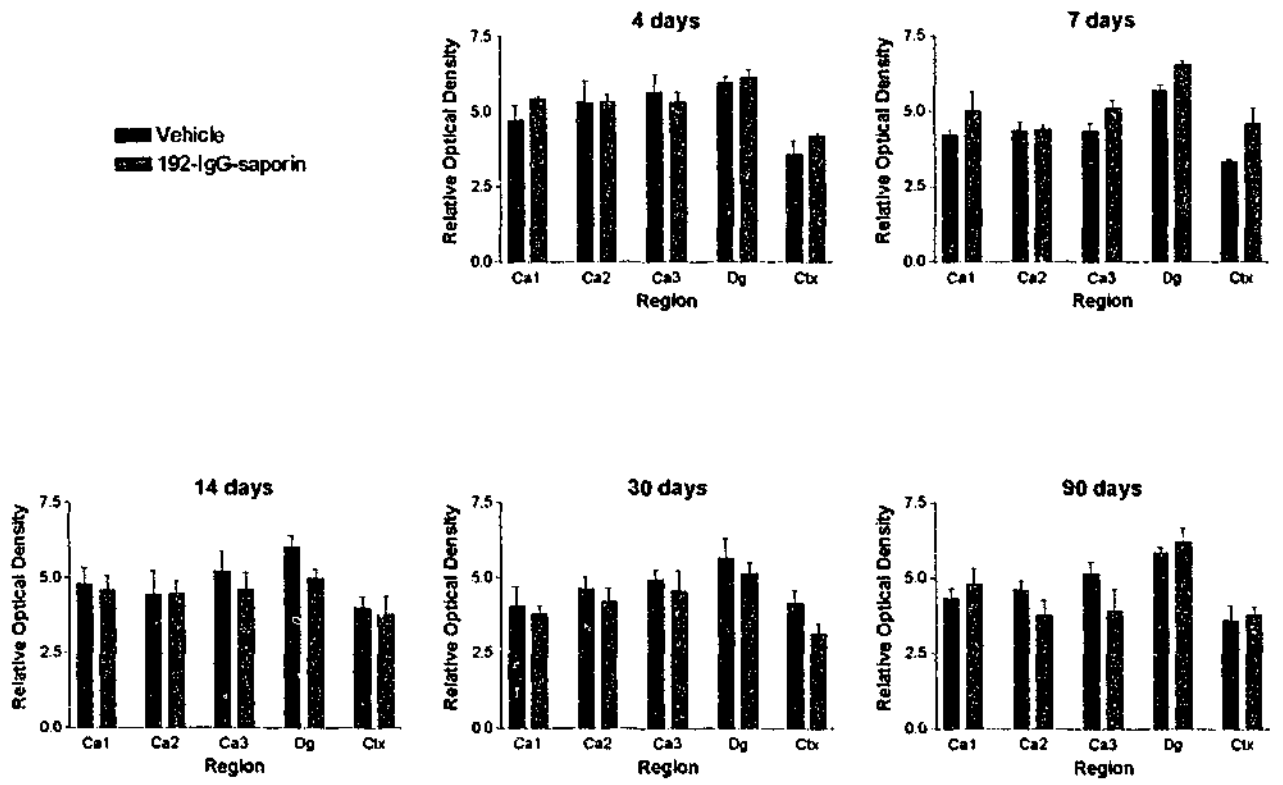


Figure 4.27. Levels of tumour necrosis factor-α-converting enzyme (TACE) mRNA in selected cortical and hippocampal regions from vehicle treated and 4, 7, 14, 30 and 90 day post-operative 192-IgG-saporin treated groups, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. The mean ROD level in the 192-IgG-saporin treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

4.4 DISCUSSION

The nearly complete loss of AChE in the cortex, hippocampus and medial septum and persistence of AChE levels in the caudate putamen and thalamic nuclei following i.c.v. injection of 192-IgG-saporin, demonstrates the selective nature of this immunotoxin for basal forebrain cholinergic neurons. Although the amygdaloid nuclei are known to receive cholinergic basal forebrain innervation, 192-IgG-saporin treatment did not significantly affect the level of AChE in this region, indicating a sparing of the amygdala-projecting basal forebrain nuclei. This is consistent with the finding that, unlike other target regions of the basal forebrain, the amygdala exhibits a striking 'decoupling' between the expression of cholinergic markers and NGF receptors including p75 (Kordower et al., 1989). The finding that only a few scattered NGF receptor immunoreactive profiles are seen in this structure has led to the hypothesis that amygdaloid-projecting basal forebrain neurons may not express the receptors for NGF (Kordower et al., 1989). The finding presented here, in relation to the lack of effect of the NGF receptor selective 192-IgG-saporin on amygdaloid AChE levels, supports this hypothesis.

It was interesting to find that there was some degree of recovery of the 192-IgG-saporin induced loss of AChE at the 30 and 90-day post-operative time points. The relationship between the magnitude of initial AChE loss and subsequent recovery and the different post-operative time points differed between regions. While the medial septum and hippocampus exhibited a gradual loss of AChE peaking at 14 days post 192-IgG-saporin treatment with, possibly, a minor recovery of AChE levels at the 30 and 90 day time points, the cortical regions examined showed the greatest reduction in AChE at the earliest, 4 day, time point and then exhibited a gradual recovery of AChE levels up to near control levels by the 90 day time point. One possible explanation for these regional differences in the response of basal forebrain innervated structures to 192-IgG-saporin treatment may be

the density of p75 receptors on individual sub-populations of basal forebrain neurons. As will be discussed in the following chapter, the basal forebrain can be subdivided into distinct groups of nuclei that differentially project to the various telencephic structures including the hippocampus and cortex. An immunohistochemical study by Kordower et al., (1989) has shown that different populations of these nuclei vary with respect to the degree of NGF receptor expression and therefore it is certainly possible that there will be some variance in the response of these neuronal populations, and consequently their target regions, to the NGF receptor immunotoxin, 192-IgG-saporin. The recovery of AChE in the basal forebrain innervated areas reported here is indicative of some degree of cholinergic basal forebrain plasticity and is consistent with a study by Gu et al., (1998) which similarly reports a region and time dependent recovery in cholinergic markers following immunotoxic 192-IgG-saporin lesions. Furthermore, Farris et al., (1993) has reported that AChE activity initially disrupted following physical transection of basal forebrain cholinergic projections can be re-established with sufficiently long survival times as a result of neuritic extension of the transacted axon toward distally denervated target areas. Interestingly, a striking up-regulation of NGF receptor immunoreactive neurons has been observed in the cortex of AD subjects, which has been hypothesised to represent cholinergic basal forebrain plasticity in response to an initial loss of these neurons (Mufson et al., 1992). As will be further discussed, recovery of cerebral cholinergic activity at the 30 and 90-day time points following cholinergic denervation may be of particular relevance in light of the finding that most of the changes in neurochemical markers seen here, as a result of 192-IgG-saporin treatment, were observed at the later 30 and 90 day time points.

Selective deafferentation of the cholinergic basal forebrain neurons had no effect on nAChR density in areas innervated by the basal forebrain. No significant difference in the level of cortical or hippocampal [^{125}I]α-BGT or [^{3}H]epibatidine binding was seen in the

192-IgG-saporin treated groups, at any time point, relative to the control group. This finding is consistent with previous studies reporting no change in the binding of selective nAChR ligands in the hippocampus and cortex following 192-IgG-saporin treatment (Rossner et al., 1995b & 1995c). Considering that nAChRs (along with muscarinic acetylcholine receptors) are likely to represent the primary cholinoceptive target for cholinergic basal forebrain transmission, it was expected that following a loss of neurotransmission there may have been some sort of homeostatic change in the level of these target receptors, similar to that seen in other neurotransmitter systems. For example, the up-regulation of dopamine receptors in the striatum in response to a significant loss of the dopaminergic projections that innervate the striatum has been well documented (for reviews see Kostrzewa, 1995; Langer et al., 1981). Furthermore, a study by Rossner et al., (1995b) has reported that there is a significant up-regulation of cortical M_1 and M_2 muscarinic receptors 7 days after a single injection of 192-IgG-saporin. Based on this finding, along with the lack of change in nAChR ligand binding reported here, one might speculate that muscarinic receptors represent the primary target for cholinergic basal forebrain neurotransmission. However, the modest but significant increase in α_4 and α_7 nAChR subunit mRNA expression in the cortex and hippocampus respectively, reported here as a result of 192-IgG-saporin treatment, indicates there may indeed be some interaction between cholinergic basal forebrain neurons and the major $\alpha_4\beta_2$ and α_7 nAChR subtypes in this area. It is worth noting that this increase in mRNA for the α_4 and α_7 subunits was not seen until the 14 day and 30/90 day time points respectively, following 192-IgG-saporin treatment. Interestingly, in terms of the AChE histochemistry, these time points correspond with an apparent recovery of cholinergic basal forebrain activity in the cortex (14 day time point) and hippocampus (30 and 90 day time points), rather then an active loss of these neurons. It is therefore possible that the up-regulation of nAChR subunit mRNA reported here may be a response to an increase in the level of ACh in these

areas resulting from the recovery of cortical and hippocampal basal forebrain projections. This would be consistent with findings showing that increased levels of nAChR activation causes an increase in the density of α_7 nAChRs in rat hippocampal neurons (Ridley et al., 2001) or human neuronal cell cultures (Molinari et al., 1998). It is perhaps not surprising that a corresponding significant increase in [^{3}H]epibatidine or [^{125}I]α-BGT binding was not seen here at the 14 or 30 and 90 day time points respectively, considering the relatively modest changes in nAChR subunit mRNA expression and that a number of reports have indicated a lack of correlation between the regulation of nAChRs at the protein and mRNA level (Marks et al., 1992; Pauly et al., 1996).

To summarise, despite a lack of change in the density of the mature receptor constructs, the up-regulation of α_4 and α_7 nAChR subunit mRNA in the cortex and hippocampus in response to 192-IgG-saporin treatment suggests that the major $\alpha_4\beta_2$ and α_7 nAChR subtypes may represent targets for cholinergic basal forebrain neurons in these areas. Furthermore, the stability of nAChR receptor levels following a nearly complete loss of cholinergic basal forebrain projections indicates that the consistently reported loss of nAChR levels in AD may not be specifically related to the degeneration of this neuronal population.

The level of expression of NR1, NR2A and NR2B NMDAR subunit mRNA was not affected until 90 days after the initial treatment with 192-IgG-saporin. At the 90-day post treatment time point, there was a moderate increase in the expression of all three subunits throughout the cortex and hippocampus, however, this result was only statistically significant for the expression of the NR2A subunit in the cortex and Ca1 field of the hippocampus. It has previously been reported that 7 days after 192-IgG-saporin treatment there is a significant decrease in NMDAR levels (Rossner et al., 1995a) and 30 days after 192-IgG-saporin treatment there is no change in the density of NMDARs (Nicolle et al., 1997). Taken together with the results reported here showing an increase in NMDAR

subunit mRNA expression 90 days after 192-IgG-saporin treatment, these findings indicate that the duration of cholinergic basal forebrain hypofunction may be an important factor in relation to changes in NMDARs in the hippocampal and cortical target regions. Furthermore, in the context of the temporal variation in AChE levels observed following 192-IgG-saporin treatment, the increase in NR2A subunit mRNA at the 90 day time point corresponds with a recovery rather than degeneration of cholinergic basal forebrain neurons. Therefore the increase in NR2A subunit mRNA in the Ca1 field of the hippocampus and cortex is likely to correspond with an increase in ACh levels in these areas following a sustained period of nearly complete deprivation of cholinergic signalling. In the Ca1 field of the hippocampus, acetylcholine can either inhibit glutamate release through the activation of presynaptic muscarinic receptors (Hounsgaard, 1978; Marchi et al., 1989) or potentiate glutamate release through the activation of nicotinic receptors (Gray et al., 1996). Therefore, the net result of cholinergic modulation of glutamate release will depend on the balance between nicotinic and muscarinic presynaptic receptor activation on glutamatergic neurons. Based on the results reported here showing a lack of change in cortical and hippocampal nAChR receptor density following 192-IgG-saporin treatment, and reports that there is a significant up-regulation of muscarinic receptors under the same conditions (Rossner et al., 1995b), one might speculate that an increase in telencephic ACh levels as a result of basal forebrain plasticity may have an inhibitory effect on glutamatergic neurotransmission. In this context, the increase in NR2A mRNA expression reported here may reflect a homeostatic response to a reduced basal level of glutamatergic transmission. Of course this is a speculative line of reasoning but offers one possible explanation for the reported results. In support of this idea, a study by Hoffmann et al., (2000) has reported that NR2A subunit mRNA expression is up-regulated following blockade of glutamatergic transmission in neuronal culture and also during neocortical development *in vivo*. In relation to the changes in NMDARs seen in AD the results

reported here do not correlate with studies on NMDAR levels in post-mortem AD brains. Alzheimer's disease subjects have been reported to exhibit a significant decline in NMDAR density (Greenamyre et al., 1987; Greenamyre et al., 1985) and subunit protein levels (Sze et al., 2001) relative to age-matched controls. Considering the results reported here show an *increase* in NMDAR subunit expression, it may be concluded that the reduction in NMDAR levels and subunit proteins seen in AD is not likely to be directly related to the degeneration of cholinergic basal forebrain neurons. Having said that, there are important limitations that should always be considered when comparing results from animal models of AD-like neurodegeneration to those generated from studies of post-mortem AD tissue. By necessity, studies on post-mortem tissue represent late stages of the disease and the loss of receptor proteins and other neurochemical markers in these preparations may be the passive result of frank neurodegeneration, which is extensive by this stage of the disease, rather then dynamic and facilitative changes in the neurodegenerative cascade. Relative to the slow progression of neurodegeneration in AD, which occurs over many years, 90 days is only a very small period of time, and it is possible that reactive changes in NMDAR mRNA and protein levels, as reported here, may occur at early stages of AD in response to cholinergic basal forebrain degeneration.

In summary, with regard to NMDARs, the work presented in this chapter indicates that, in addition to the changes in NMDARs seen at the receptor level in AD, there may also be changes at the subunit mRNA level as a result of extended periods of cholinergic basal forebrain denervation. The selective up-regulation of NR2A NMDAR subunit mRNA suggests that NR2A containing NMDAR subtypes may be important targets for the regulation of cortical and hippocampal glutamatergic neurotransmission by basal forebrain cholinergic activity. Such receptors may be interesting targets for AD therapy considering the involvement of both NMDARs and cholinergic basal forebrain neurons in cognitive function. Clearly, a useful direction for future work in this area will be to compare the

findings reported here in relation to NMDAR mRNA levels with NMDAR protein levels under the same conditions using immunohistochemical or radioligand binding techniques.

A study by Jouvenceau et al., (1997) reports a significant increase in excitatory post-synaptic potentials (EPSPs) elicited in the Ca1 field of the hippocampus 2 weeks after cholinergic denervation with 192-IgG-saporin. Specifically, electrophysiological examination of Ca1 pyramidal cells in rat hippocampal slices showed that, in response to glutamatergic stimulation, there was a significant potentiation in the amplitude of the EPSP recorded from 192-IgG-saporin treated rats which was suggested to represent an enhanced synaptic efficacy of glutamatergic synapses in these animals. In an attempt to discover the underlying mechanism for this finding, Jovenceau *et al* (1997) also report, that the increase in EPSPs was not related to an increase in NMDA receptor density, a change in affinity of the NMDA receptors for their endogenous ligand, or a change in the presynaptic calcium-dependent mechanisms that control glutamate release. The authors argued however, that the increase in EPSPs in 192-IgG-saporin treated rats could be related to an increase in glutamate release mediated by calcium-*independent* mechanisms. One such mechanism may include the enhancement of glutamate release through the activation of presynaptic guanylate cyclase and the subsequent production of cGMP by nitric oxide as discussed previously (fig 2.2). Consistent with this hypothesis, it is reported here that there is a significant increase in the expression of nNOS in the dentate gyrus and Ca1 field of the hippocampus 14 days after treatment with 192-IgG-saporin. In the dentate gyrus this effect was also seen at 7 days after 192-IgG-saporin treatment and persisted up until the 90 day post-operative time group. An increase in nNOS expression may lead to an increase in neuronal nitric oxide production within glutamatergic neurons and consequently an enhancement of EPSPs and synaptic plasticity in the manner described for the induction of LTP. Collingridge, (1992) have reported that induction of LTP occurs in the dentate gyrus and Ca1 field of the hippocampus and is dependent on NMDAR activation. Considering

that ACh facilitates glutamatergic transmission in the cortex and hippocampus (Markram et al., 1990a; Markram et al., 1992; Markram et al., 1990b), a loss of basal forebrain innervation of these areas may reasonably be expected to be associated with glutamatergic hypofunction. Furthermore, the recovery of basal forebrain cholinergic neurotransmission may also contribute to cortical and hippocampal glutamatergic hypofunction due to the activation of an increased number of inhibitory presynaptic muscarinic receptors associated with the initial loss of cholinergic neurons, as discussed previously. The up-regulation of the neuronal machinery involved in LTP of glutamatergic synapses, such as NMDARs and nNOS, may therefore represent a homeostatic mechanism in response to a loss of glutamatergic transmission. O'Mahony et al., (1998) have demonstrated that cholinergic basal forebrain denervation correlates with enhanced nNOS activity in the cortex, which can be inhibited by NMDAR blockade. This finding shows that the increased nNOS activity caused by a loss of cholinergic basal forebrain neurons is NMDAR mediated (as would be expected based on the described model for LTP) and is consistent with the increase in EPSPs reported by Jouvenceau et al., (1997) and the increase in nNOS mRNA expression reported here following 192-IgG-saporin treatment. Based on studies which have shown that increased levels of NO can cause excitotoxic neuronal damage (Dawson et al., 1991; Loiacono et al., 1992; Przedborski et al., 1996; Schulz et al., 1996), an increase in hippocampal and cortical nNOS activity caused by cholinergic basal forebrain degeneration may have implications for the susceptibility of these regions to excitotoxic neuronal degeneration. As has been discussed, the cortex and hippocampus are among the earliest and most severely effected regions in AD in terms of neurodegeneration. A study by Simic et al., (2000) has shown that in the hippocampus and cortex of AD subjects, but not age-matched controls, there is a significant correlation between the expression of nNOS, neuronal loss and markers for neuronal degeneration such as microglial invasion.

In conclusion, with regard to nNOS expression, the results presented here indicate that the significant loss of nNOS expressing neurons in AD subjects reported by Thorns et al., (1998) is not likely to be directly related to the degeneration of the cholinergic basal forebrain system. Of course, as outlined above, in the context of AD-like neurodegeneration this study has only looked at a limited and relatively early set of time points. Therefore, it is possible that changes in neurochemical markers, such as nNOS, seen in AD brains may reflect responses to more extended periods of cholinergic denervation and associated pathology. Based on the neurotoxic effects of excessive nNOS stimulation, it is possible that the increase in nNOS expression associated with cholinergic basal forebrain degeneration, seen here, may contribute to further neuronal damage in the hippocampus and cortex in AD. Future directions in research in this area may include the correlation of markers for neuronal degeneration in hippocampus and cortex with the increase in nNOS expression associated with cholinergic basal forebrain loss.

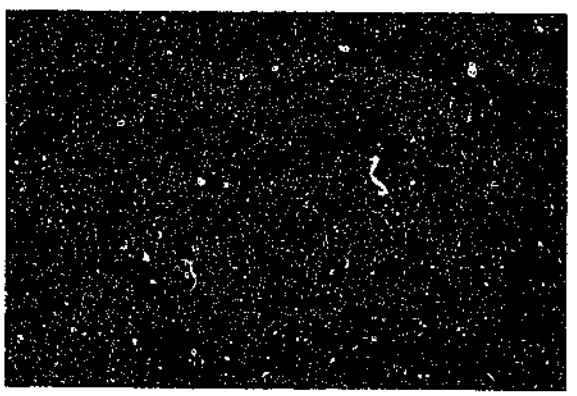
Based on evidence that the cholinergic stimulation plays an important role in regulating the processing and expression of APP (for review see Rossner et al., 1998), this study has examined the effect of 192-IgG-saporin treatment on the expression of APP695 and a number of proteins involved in its proteolytic processing including BACE, PS1, PS2 and TACE. Using human embryonic kidney cells, it was first demonstrated by Nitsch et al., (1992) that stimulation of the M_1 and M_2 muscarinic receptor subtypes facilitates the secretory processing of APP. Muscarinic receptor stimulation was found to be associated with an increase in the release of the soluble N-terminal domain of APP and a decrease in the amount of full length membrane-associated APP. This effect has also been demonstrated in rat brain slices by Farber et al., (1995) and is dependent on protein kinase (PKC) activation (Buxbaum et al., 1990; Slack et al., 1993). Furthermore, studies by Kim et al., (1997) and Kuisak et al., (1999) have reported that nAChR stimulation can also mediate the processing of APP. Based on these findings it may be reasonably expected that

the degeneration of the cholinergic basal forebrain system associated with AD pathology could significantly affect APP processing in the cortical and hippocampal target regions. Consistent with this hypothesis, Rossner et al., (1997) has reported a significant decrease in the level of secreted APP fragments and a concomitant increase in membrane bound APP in response to 192-IgG-saporin induced cholinergic basal forebrain lesions. The mechanism underlying this effect is not known but is clearly suggestive of a reduction in cholinergic mediated activity of the proteolytic secretase enzymes that process APP. The results presented in this chapter demonstrate that a reduced level of secretase activity in response to cholinergic basal forebrain degeneration is not likely to be caused by changes in the expression of BACE, PS1, PS2 or TACE at the mRNA level. Relative to vehicle treatment, i.c.v injection of 192-IgG-saporin did not significantly affect BACE, PS1, PS2 or TACE mRNA levels in the hippocampus or cortex at various time points up to 90 days after treatment. Of course it has not been conclusively demonstrated whether or not that PS1, PS2 and TACE are in fact APP cleaving secretases. It is possible that cholinergic basal forebrain denervation induces changes in the expression of, as yet, unidentified secretase enzymes. Furthermore, it is also possible that changes in the level of APP processing are mediated by changes in the activity of existing secretases rather then at a transcriptional level. Although no change in BACE, PS1, PS2 or TACE was seen, it has been reported here that there is a significant increase in the expression of APP695 as a result of 192-IgG-saporin treatment at the 90-day post-injection time point. This finding is consistent with studies by Lin et al., (1998) and Leanza, (1998b) reporting that 6 months after i.c.v. there is a significant up-regulation of APP protein levels of up to 71% as assessed by immunohistochemistry. Importantly, the work presented here demonstrates some degree of both regional and temporal specificity associated with the up-regulation of APP mediated by cholinergic basal forebrain degeneration. The increase in APP was localised to the cortex and was not seen until 3 months after the initial 192-IgG-saporin

treatment. Together with reports that at 6 months after 192-IgG-saporin treatment APP levels are significantly up-regulated in the hippocampus as well as the cortex (Leanza, 1998b; Lin et al., 1998), this finding demonstrates that a loss of cholinergic basal forebrain neurons leads to an up-regulation of APP in the corresponding target areas which is progressive and persistent. In terms of the relevance of this finding with regard to AD pathology, it is possible that an increase in APP levels in these regions may be responsible for increased Aβ production and subsequent plaque formation. It has previously been reported that over-expression of APP in transgenic mice leads to progressive Aβ deposition and cognitive disturbance with increasing age (Games et al., 1995; Hsiao et al., 1996; Nitsch, 1996). A great deal of work in the field of AD research has been dedicated to establishing a causal relationship between the increased Aβ load and loss of basal forebrain cholinergic neurons seen in AD patients. Collectively, the findings that cholinergic basal forebrain degeneration leads to increased APP levels and that over-expression of APP leads to increased Aβ deposition, support the hypothesis that degeneration of the cholinergic basal forebrain system is a causative factor in relation to subsequent AD-related neurodegeneration. As a direction for further work in this area, it would be interesting to investigate whether the increase in APP associated with cholinergic basal forebrain denervation can be attenuated by cholinergic replacement strategies such as chronic stimulation of muscarinic or nicotinic receptors in the hippocampus and cortex.

Chapter 5

The effect of excitotoxic lesioning of the basal forebrain on multiple neurochemical markers in the rat brain



5.1 INTRODUCTION

In addition to providing cholinergic innervation, the basal forebrain system is also the source of a significant amount of non-cholinergic projections to various telencephic regions including the hippocampus and cortex. Approximately 50% of the hippocampal and 15 – 20% of the neocortical projections are non-cholinergic (Gritti et al., 1997; Rye et al., 1984). The majority of these non-cholinergic neurons are ascending GABAergic projections that synapse predominately on GABAergic interneurons within the hippocampus and cortex (Brauer et al., 1993; Brauer et al., 1991; Freund et al., 1988; Gulyas et al., 1990; Kiss et al., 1990). The cholinergic basal forebrain projections, however, synapse primarily on the dendritic arbours of pyramidal and dentate granule cell neurons and to a lesser extent on GABAergic interneurons (Beaulieu et al., 1991; for reviews see Butcher, 1995; Wainer et al., 1990; Wainer et al., 1993). Glutamatergic neurons are likely to represent the major hippocampal and cortical targets for cholinergic and GABAergic modulation of neurotransmission in these regions (Flint et al., 1985; Lamour et al., 1982; Reine et al., 1992). Both cholinergic and GABAergic basal forebrain transmitter systems may facilitate neurotransmission in the hippocampus and cortex through different mechanisms – directly for cholinergic inputs and through disinhibitory mechanisms for GABAergic inputs (fig. 5.0). Duque et al., (2000) have reported that the firing rate of both GABAergic and cholinergic basal forebrain neurons is correlated with cortical electroencephalographic activation.

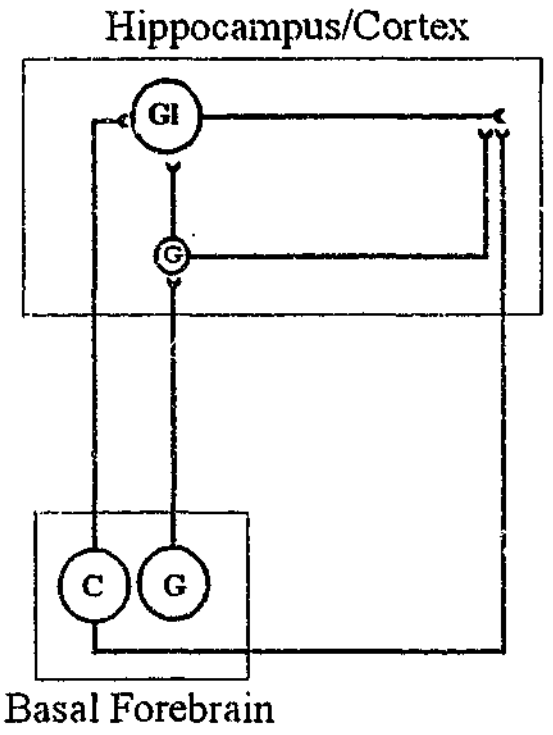


Figure 5.0. Schematic representation of the ascending cholinergic (C, ●) and GABAergic (G, ●) basal forebrain pathway with respect to synaptic targets in the hippocampus and cortex. Cholinergic projections synapse heavily on the dendritic arbours of pyramidal and dentate granule neurons, while GABAergic projections synapse primarily on GABAergic interneurons. Activation of both neuronal systems will have predominately excitatory effect on target neurons in the hippocampus and cortex. A significant percentage of these target neurons are likely to be glutamatergic (Gl, ●).

Before toxins that selectively target cholinergic basal forebrain neurons such as 192-IgG-saporin became available, attempts to model the cholinergic basal forebrain degeneration seen in AD were based around the use of basal forebrain injections of various excitotoxins. As a consequence of the non-selective nature of these excitotoxic agents with regard to neuronal type, both cholinergic and GABAergic forebrain neurons are destroyed (Abdulla et al., 1994; Dunnett et al., 1987). Based on the abundance of evidence showing a loss of cholinergic forebrain neurons in AD, and relatively little information regarding GABAergic basal forebrain neurons, it has generally been accepted that cholinergic nuclei are selectively affected by the neurodegenerative process. However, definitive and consistent evidence regarding the integrity of GABAergic basal forebrain neurons following the widespread neurodegeneration associated with AD has not been extensively published. Although it has been demonstrated that glutamic acid decarboxylase (GAD) levels are relatively unaffected in AD (Reinikainen et al., 1988), GAD is expressed in a variety of neuronal types and also glial cells (Martinez-Rodriguez et al., 1993) and

therefore has limited use as a definitive marker for GABAergic neurons. In cortical regions from post-mortem AD tissue, a significant reduction in the level of GABA has been reported (Palmer, 1996; Rossor et al., 1982). It has also been reported that there is a significant reduction in benzodiazapine binding to GABA receptors in various cortical and hippocampal regions in AD brains (Jansen et al., 1990). Although these findings do not directly implicate the involvement of basal forebrain GABAergic neurons, they do demonstrate that GABAergic neurons may be susceptible to degeneration in AD. While the degeneration of the cholinergic basal forebrain neurons in AD is now a well established finding and consequently the focus of a great deal of research; based on the current understanding of the complicated pattern of neurodegeneration seen in AD, it is perhaps premature to rule out the involvement of GABAergic basal forebrain neurons.

Compared with the selective lesioning of cholinergic basal forebrain using 192-IgG-saporin, non-specific excitotoxic lesioning of the basal forebrain neurons has generally been a far more successful method for simulating the cognitive deficits seen in AD (Altman et al., 1985; Connor et al., 1991; Dunnett et al., 1987; Marston et al., 1993; Miyamoto et al., 1985; Robbins et al., 1989a; Robbins et al., 1989b). Furthermore, excitotoxic agents such as α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) which produce the greatest cholinergic cell loss are associated with less cognitive impairment than other excitotoxins which produce relatively less cholinergic damage. In selective basal forebrain lesions using 192-IgG-saporin, (Waite et al., 1995) have reported that substantial cholinergic loss (as assessed by AChE levels) of at least 90% are required to produce cognitive deficits. Similar cognitive impairments seen as a result of excitotoxic basal forebrain lesions are associated with relatively modest reductions in AChE activity of around 20 – 30% (Gutierrez et al., 1999). Collectively, data comparing behavioural effects to the relative degree and selectivity of cholinergic basal forebrain lesioning has lead many authors to question the specific involvement of cholinergic basal forebrain

neurotransmission in certain aspects of cognitive function and consequently the relevance of the degeneration of this neuronal system to the clinical symptoms associated with AD (Dunnett et al., 1987; Robbins et al., 1989b; for review see Dunnet et al., 1991). A comparison of data generated from selective cholinergic forebrain lesions using agents such as 192-IgG-saporin, with results from the non-selective excitotoxic lesioning of basal forebrain neurons is a useful approach in attempts to outline the relative importance of cholinergic and non-cholinegic neurons in regard to the effects of the degeneration of the basal forebrain structure. In this context, Gutierrez et al., (1999) report that following a 97% reduction in cortical and hippocampal AChE levels caused by 192-IgG-saporin treatment and a 28% reduction caused by basal forebrain NMDA injections, the NMDA treated group showed a significantly greater impairment in the capacity to form new memories relative to control rats.

The aim of the work presented in this chapter has been to extend on the findings from behavioural experiments that have investigated the relative importance of cholinergic and non-cholinergic basal forebrain neurons with regard to cognitive function. Specifically, it describes the assessment of the effect of excitotoxic basal forebrain lesions on the expression of the various neurochemical markers examined in response to 192-IgG-saporin treatment, as documented in the previous chapter. By comparing results from the 192-IgG-saporin experiments with those presented here, it may be possible to identify and discuss the relative importance of cholinergic and non-cholinergic basal forebrain innervation of the hippocampus and cortex in regard to the expression of a number of neurochemical markers that may be important in AD.

The basal forebrain area of the brain is further subdivided into a number of specific regions containing groups of nuclei that differ with respect to the topographic organization of their ascending neuronal projections (fig 5.1). The medial septum (MS) and vertical limb of the diagonal band of Broca (VDB) give rise mainly to hippocampal as well some neocortical projections, while the horizontal limb of the diagonal band of Broca (HDB) projects to the olfactory bulb. The nuclei contained within the nucleus basalis of Meynert (nbM) give rise to topographically organised projections to the neocortex and amygdaloid nuclei and, to a lesser extent, to certain thalamic nuclei. The nbM is believed to be homologous to the magnocellular basal nucleus (MBN) in the rat basal forebrain. Useful reviews on the organization and connectivity of major cerebral cholinergic systems have been published by Wainer et al., (1990 & 1993) and (Butcher, 1995).

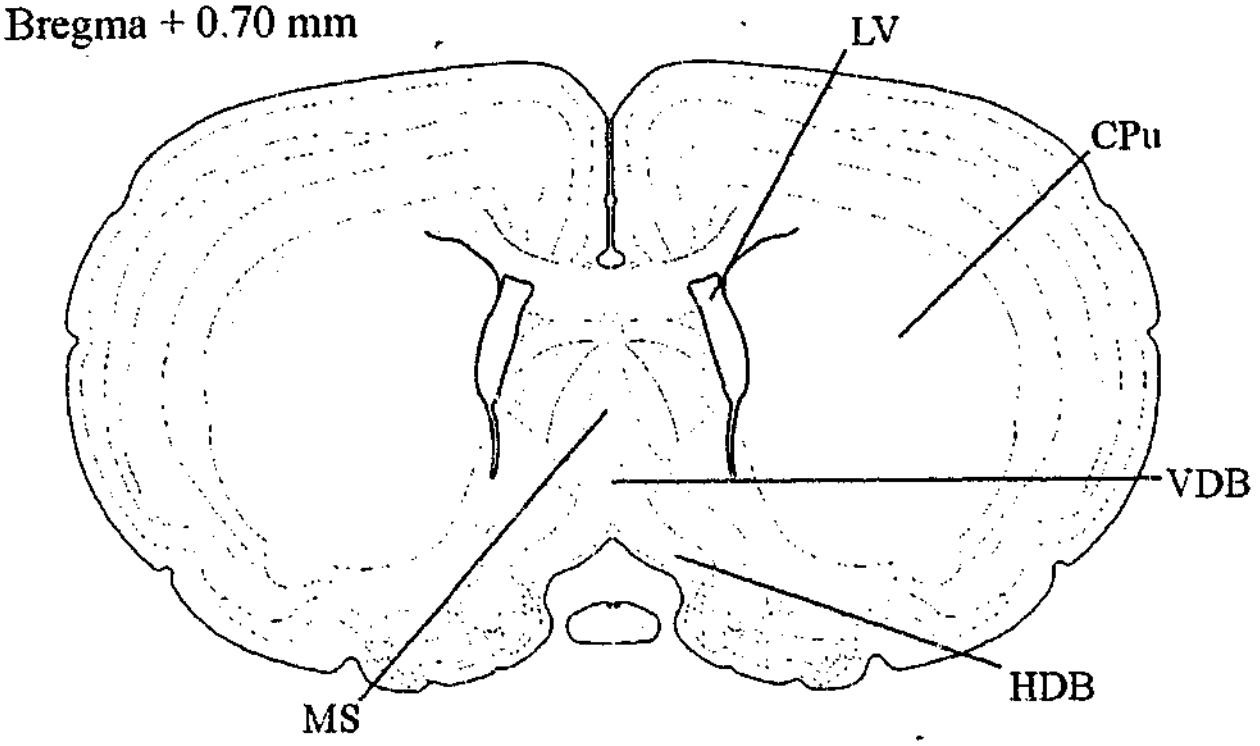


Figure 5.1. Schematic representation of coronal sections of rat brain showing the major subdivisions of the cholinergic basal forebrain system, including: the horizontal (HDB) and vertical limbs (VDB) of the diagonal band of Broca; the medial septum (MS); and the magnocellular basal nucleus (MBN). Abbreviations: 3V, 3rd ventricle; CPu, caudate putamen; LGP, lateral globus pallidus; LV lateral ventricle; SI, substantia inominata. Adapted from the atlas of Paxinos and Watson (1986).

The excitotoxic lesioning of basal forebrain nuclei was achieved through a single injection of 2,3-Pyridinedicarboxylic acid (quinolinic acid) into the magnocellular basal

nucleus (MBN) region of the rat basal forebrain. The nbM (MBN in rats) is often described as the basal forebrain region that shows the greatest degree of degeneration in AD and the loss of cholinergic nbM neurons has been correlated with cognitive impairment (Bartus et al., 1982; Price, 1986). This chapter presents the effect of excitotoxic MBN lesions on the cortical and hippocampal expression of nicotinic and glutamatergic markers as well as APP695 and other proteins involved in the processing of APP. These results are discussed and compared to the results documented in the previous chapter describing the effect of selective cholinergic lesioning of the basal forebrain on the same neurochemical markers.

5.2 METHODS

5.2.1 TREATMENT OF ANIMALS AND TISSUE PREPARATION

Sprague-Dawley rats (250 – 300 g) of either sex were anaesthetised with pentobarbitone (60 mg/kg i.p.) and constrained to a tilted skull position (-0.3 mm) using a Kopf stereotaxic apparatus. A small hole was drilled into the skull and a stainless steel 30 gauge blunt tipped injection cannula was inserted into the MBN at the following stereotaxic coordinates: 1.40 mm caudal to bregma, 2.70 mm left from the midline and 7.40 mm ventral from the cortical surface according to the atlas of Paxinos and Watson (1986). Injections of 1 µl of either 100 nmol of quinolinic acid in PBS (pH 7.4) or PBS (pH 7.4) alone (vehicle) were delivered at the rate of approximately 0.2 µl/min. The experimental paradigm therefore consisted of two groups, with 4 animals receiving injections of quinolinic acid and four animals receiving injections of vehicle. The cannula was left in place for a further 10 min following injection to allow adequate diffusion of the injected contents from the injection site. Following a recovery period of 7 days, animals were decapitated and the brains frozen over liquid nitrogen. For histochemical and autoradiographic analysis, a Reichert Jung cryostat was used to collect a series of 14 µm sections. Consecutive coronal sections were taken at 3.6 mm caudal to bregma and thaw mounted on poly-L-lysine coated slides. All tissue was stored at -70°C until required.

5.2.2 AChE Histochemistry

AChE levels were quantified using a modification of the method previously described by Koelle et al., (1949). This method has been described in detail under Chapter 2, General Methodology, *2.4 AChE histochemistry.*

5.2.3 In Situ Hybridisation Histochemistry

The protocol for in situ hybridisation is based on the methods described Wisden et al., (1994) and adapted by Loiacono et al., 1999), and has been described in detail under Chapter 2, General Methodology, *2.1 In Situ Hybridisation Histochemistry.*

5.2.4 [^{3}H]Epibatidine Autoradiography

The protocol for [^{3}H]epibatidine autoradiography was based on that described by Perry et al., (1995b) and has been described in detail under Chapter 2, General Methodology, *2.2 [^{3}H]Epibatidine Autoradiography.*

5.2.5 [^{125}I]α-Bungarotoxin Autoradiography

The protocol for [^{125}I]α-BGT was based on that used by Whiteaker et al., (1999) and has been described in detail under Chapter 2, General Methodology, *2.3 [^{125}I]α-Bungarotoxin Autoradiography.*

5.2.6 *QUANTITATION*

Images generated through the *in situ* hybridisation histochemistry, histochemical and binding protocols were digitally captured and selected anatomical regions were quantified using Scion imaging software. For mRNA images, [^{125}I]α-BGT autoradiographs and AChE histochemical stains the system was calibrated against a set of graded greyscales (Kodak), allowing for quantitation in terms of relative optical density (ROD). For [^{3}H]epibatidine autoradiographs, the system was calibrated against a set of standard tritium scales, allowing for quantitation in terms of nCi/mm^2. Differences in ROD or nCi/mm^2 between quinolinic acid and vehicle treated groups were assessed statistically using an unpaired student's t test. Statistical significance was signified by P values of less then 0.5. Values reported here represent the mean of measurements taken from 4 animals. Issues relating to quantitation have been described in greater detail in Chapter 2, General Methodology, *2.5 Quantitation.*

5.3 RESULTS

5.3.1 AChE HISTOCHEMISTRY

To assess the degree and regional specificity of cholinergic degeneration following quinolinic acid injection, AChE levels were quantified through densitometric analysis of the dentate gyrus, Ca1, Ca2 and Ca3 regions of the hippocampus, and cortical layers 1-3 and 4-6 (Paxinos et al., 1986). Representative AChE stained sections from both quinolinic acid and vehicle treated animals are shown in figure 5.2. (cortex and hippocampus; bregma – 3.6 mm) and figure 5.3 (caudate putamen; bregma + 0.70 mm).

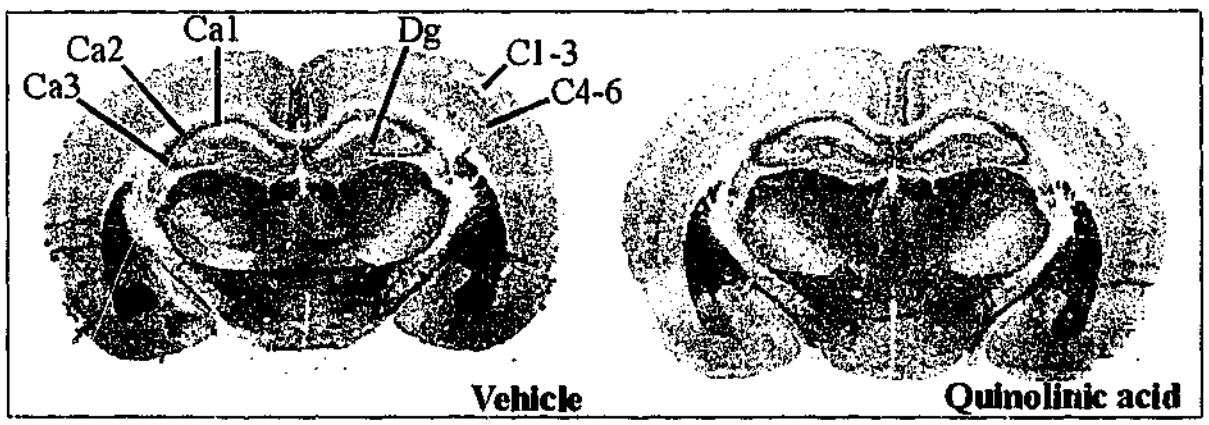


Figure 5.2. AChE histochemistry in representative coronal sections approximately 3.6 mm caudal to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus. Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3, C4-6, cortical layers 4-6; Dg, dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

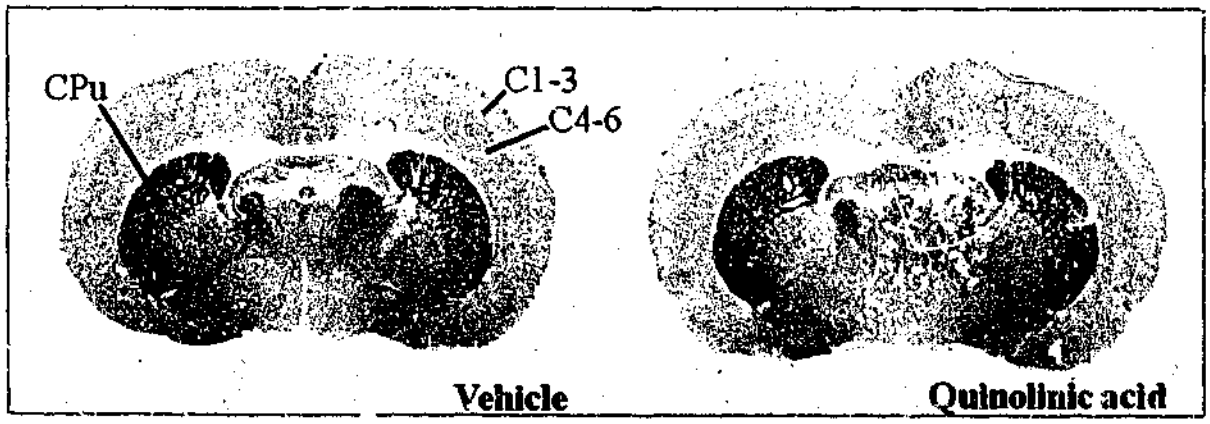


Figure 5.3. AChE histochemistry in representative coronal sections approximately 0.70 mm rostral to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus. Abbreviations: C1-3, cortical layers 1-3, C4-6, cortical layers 4-6; CPu, caudate putamen. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Rats that received quinolinic acid injections into the MBN exhibited a significant loss in AChE levels of 32% and 26% in the outer (layers 1 – 3) and deep (layers 4 – 6) cortical layers respectively (fig 5.4). No significant difference between the vehicle and quinolinic acid groups was detected with respect to AChE staining in the caudate putamen or the hippocampal regions quantified.

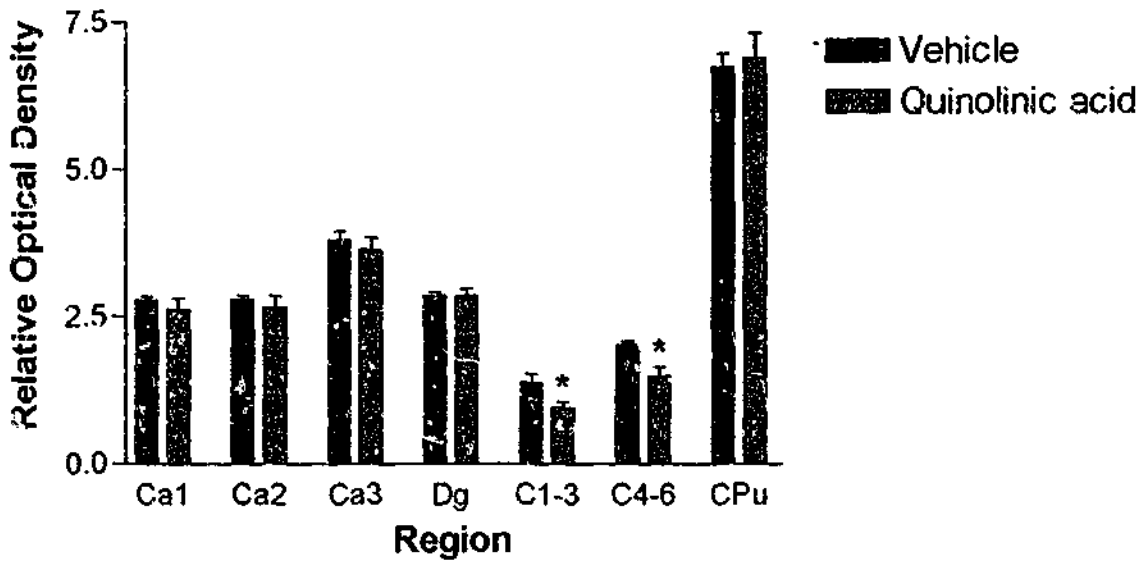


Figure 5.4. AChE levels in selected regions of coronal sections from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4-6, cortical layers 4-6; CPu, caudate putamen; Dg, dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD level in the quinolinic acid treated group was compared to the mean level of ROD in the vehicle group using an unpaired student's t test (*, $P < 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

5.3.2 nAChR SUBUNIT mRNA EXPRESSION

The level of nAChR subunit mRNA expression was examined by *in situ* hybridisation histochemistry in coronal sections from rats, 7 days after the injection of either vehicle or quinolinic acid into the MBN. Figure 4 shows the unique anatomical distribution and intensity of α_4, α_7 and β_2 nAChR subunit mRNA expression in various telencephic regions in both treatment groups.

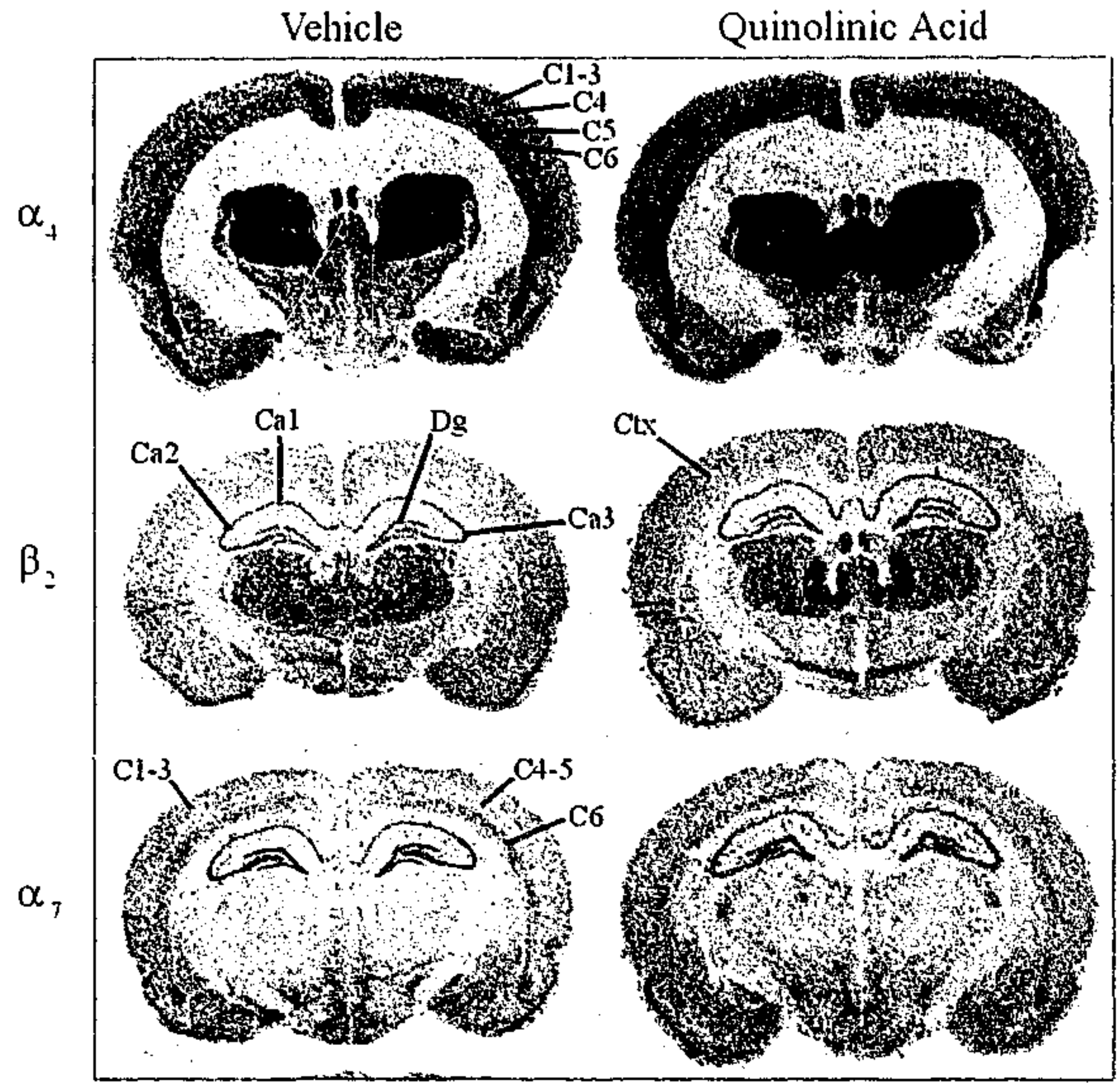


Figure 5.5. Distribution of nAChR α_4, α_7, and β_2 mRNA subunit expression in representative coronal sections approximately 3.6 mm caudal to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus. Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4, cortical layer 4; C4-5, cortical layers 4-5; C5, cortical layer 5; C6, cortical layer 6; Ctx, cortex; Dg, dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Relative to vehicle treatment, rats treated with acute injection of quinolinic acid into the MBN exhibited a small, but significant, increase in the expression of α_4, α_7 and β_2 nAChR mRNA in specific hippocampal and cortical regions (fig. 5.6). Specifically, the following changes were observed: α_4 mRNA expression was increased by 29 – 50% throughout the cortex with this effect being statistically significant in cortical layers 1 – 3 and cortical layer 5; α_7 mRNA was significantly up-regulated selectively in the Ca1 and

Ca2 fields of the hippocampus by 25 – 29%; and β_2 subunit mRNA was significantly elevated throughout all hippocampal and cortical regions quantified by 20 – 43%.

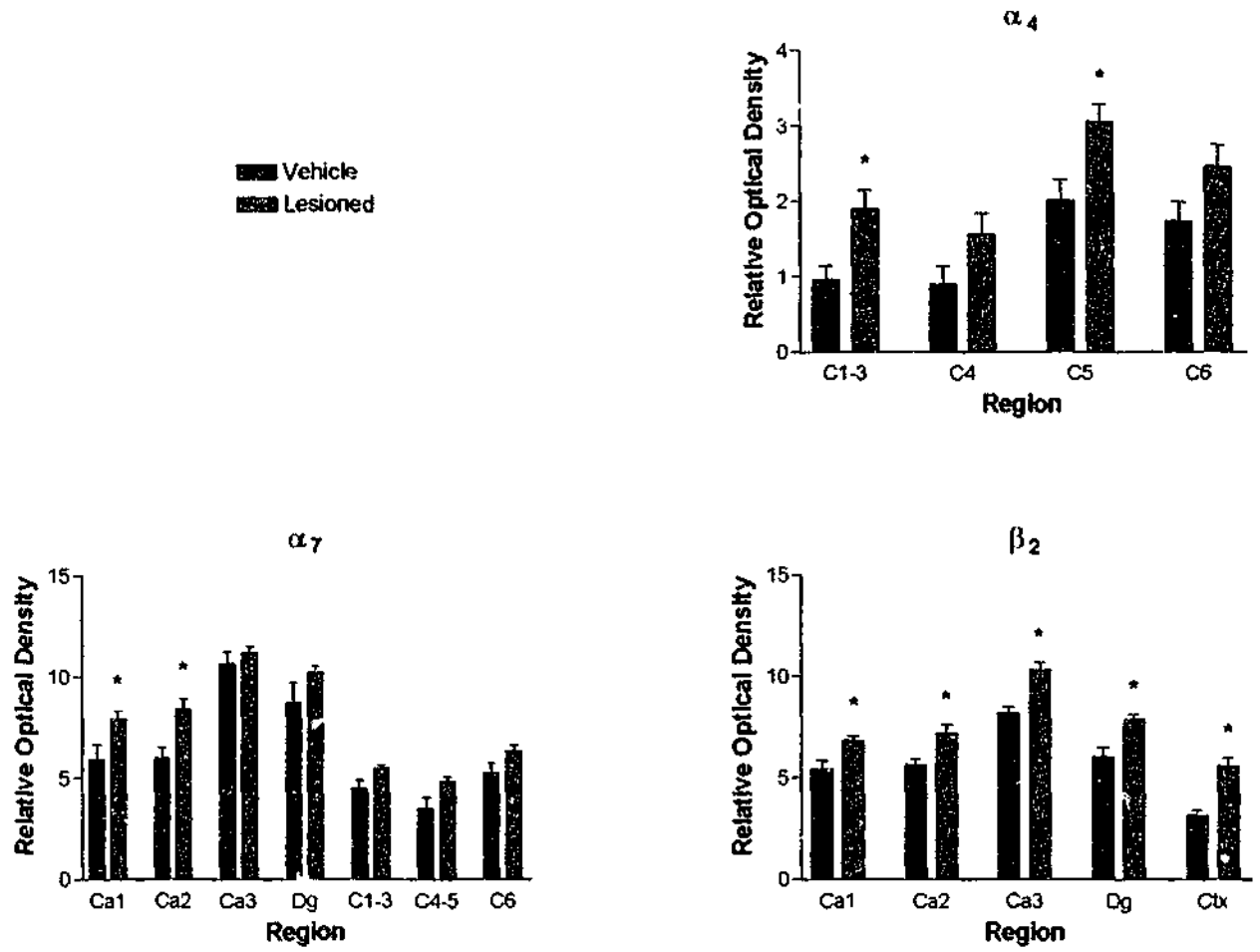


Figure 5.6. Levels of α_4, α_7, and β_2 nAChR subunit mRNA expression in selected regions of coronal sections approximately 3.6 mm caudal to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; C1-3, cortical layers 1-3; C4, cortical layer 4; C4-5, cortical layers 4-5; C5, cortical layer 5; C5, cortical layer 5; C6, cortical layer 6. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD level in the quinolinic acid treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test (*, $P < 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

5.3.3 [^{3}H]Epibatidine Autoradiography

Nicotinic receptor levels were assessed in rats from treated with an injection of either vehicle or quinolinic acid, by [^{3}H]epibatidine autoradiography. Epibatidine binds predominately to nAChRs of the $\alpha_4\beta_2$ subtype and also, to a lesser extent, to other subtypes

such as the $\alpha_3\beta_2$ receptor, which are expressed at much lower levels. The regional distribution of [^{3}H]epibatidine binding in a representative section is shown in figure 5.7. Non-specific binding, as defined by 300 μM (−)-nicotine, was undetectable in all cases (data not shown).

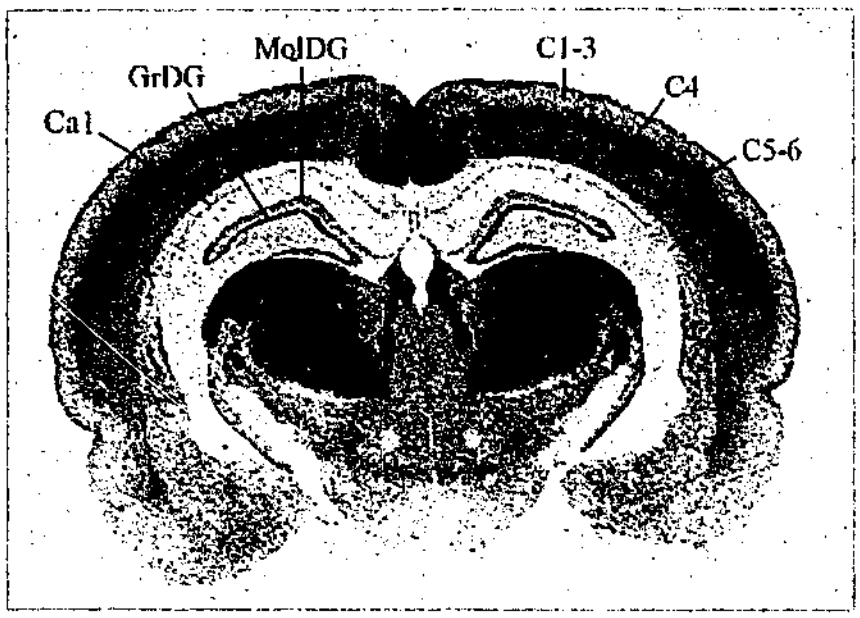


Figure 5.7. Total [^{3}H]Epibatidine binding in a representative coronal section approximately 3.6 mm caudal to bregma. Abbreviations: C1-3, cortical layers 1-3; C4, cortical layer 4; C5-6, cortical layers 5-6; GrDG, granular layer dentate gyrus; MolDG, molecular layer dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Following a single injection of quinolinic acid into the MBN, there was no significant change in the level of [^{3}H]epibatidine binding, relative to vehicle injected animals, in any of the regions examined in this study (fig 5.8).

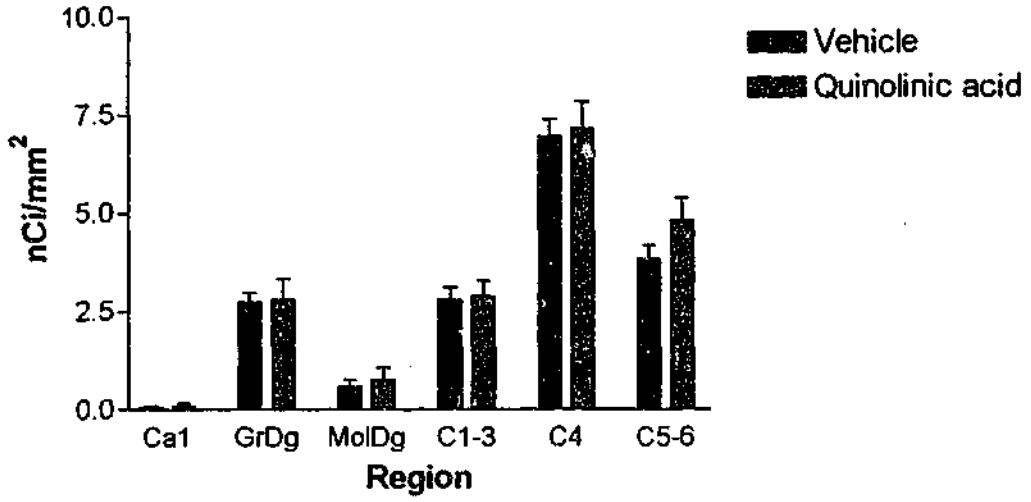


Figure 5.8. Specific [^{3}H]Epibatidine binding in selected regions of coronal sections approximately 3.6 mm caudal to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus, quantified in terms of nCi/mm^2. Abbreviations: C1-3, cortical layers 1-3; C4, cortical layer 4; C5-6, cortical layers 5-6; GrDG, granular layer dentate gyrus; MolDG, moleculer layer dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean nCi/mm^2 level in the quinolinic acid treated group was compared to the mean level of nCi/mm^2 in the vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

5.3.4 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY

The density of α_7 nAChRs in vehicle and quinolinic acid injected rats was assessed in the Ca1, Ca2, and Ca3 fields of the hippocampus, the hippocampal fissure, the stratum radiatum area of the hippocampus and cortical layers 1-5 and 6 as defined in the atlas of Paxinos and Watson (1986) by receptor autoradiography using the α_7 nAChR selective ligand [^{125}I]α-BGT. The regional distribution and intensity of [^{125}I]α-BGT binding to α_7 nAChRs in representative coronal sections from both treatment groups is shown in figure 5.9. [^{125}I]α-BGT binding was seen throughout the hippocampus and cortex, with the signal being particularly strong in the dentate gyrus. Non-specific binding, as defined by 10 mM (−)-nicotine, ranged from <10% in the dentate gyrus to approximately 40% in the stratum radiatum hippocampus (data not shown) and was subtracted from the total signal during quantitation.

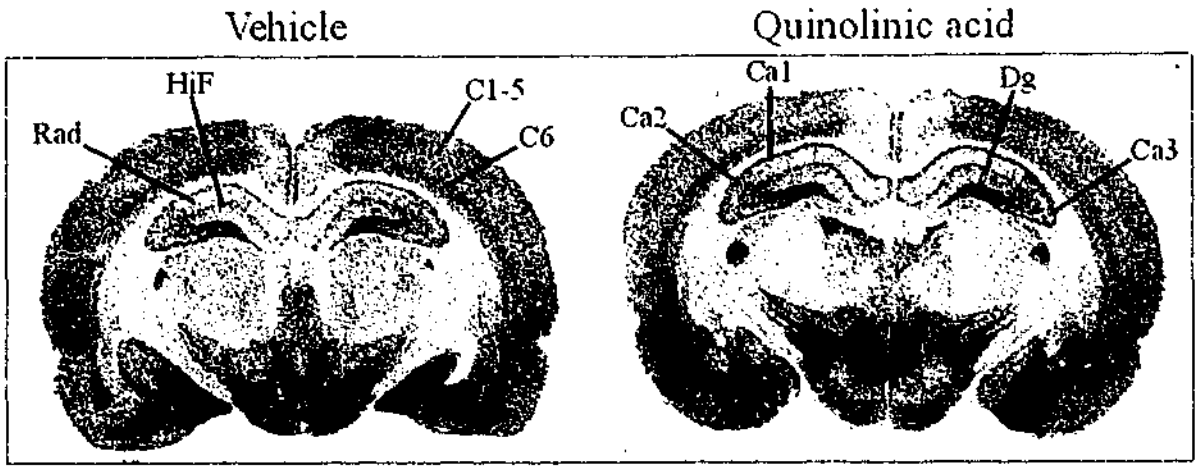


Figure 5.9. Total [^{125}I]α-BGT binding in representative coronal sections approximately 3.6 mm caudal to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus. Abbreviations: Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Specific [^{125}I]α-BGT binding was significantly increased by 29%, 39% and 23% in the Ca1 and Ca2 fields of the hippocampus and the hippocampal fissure respectively as a result of excitotoxic lesioning of the MBN region in the basal forebrain (figure 5.10). No change in specific [^{125}I]α-BGT binding was seen in the dentate gyrus, stratum radiutum hippocampus or cortex in quinolinic acid treated rats, relative to rats injected with vehicle.

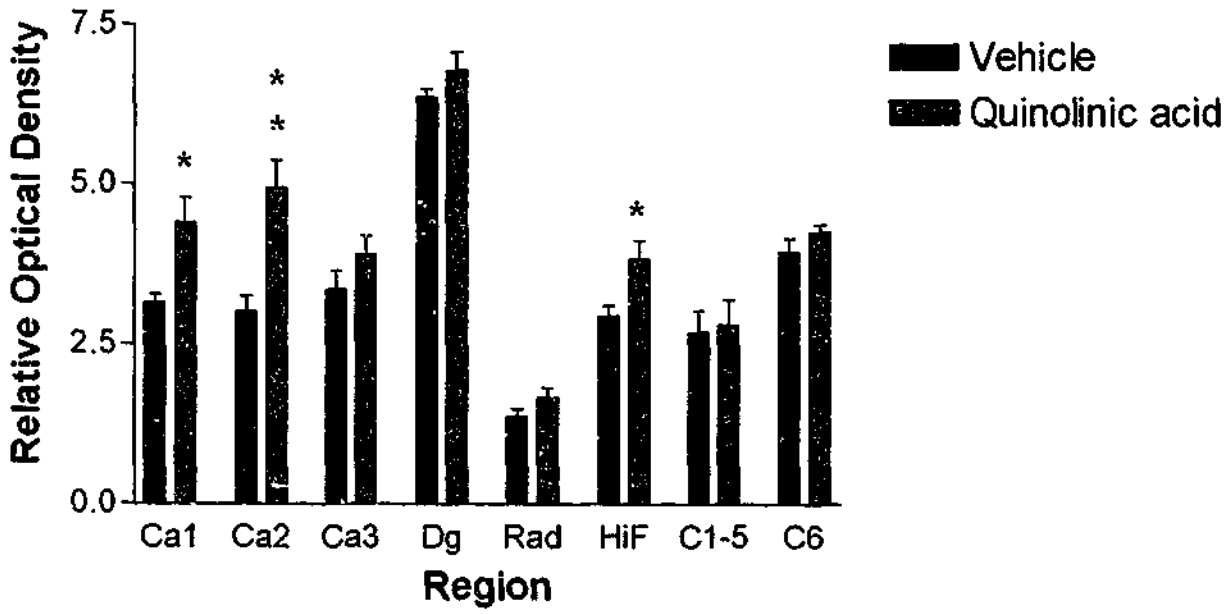


Figure 5.10. Levels of specific [^{125}I]α-BGT binding in selected regions of coronal sections approximately 3.6 mm caudal to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus;C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD level in the quinolinic acid treated group was compared to the mean level of ROD in the vehicle group using an unpaired student's t test (*, $P < 0.05$; **, $P < 0.01$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

5.3.5 NMDA Receptor Subunit mRNA Expression

NMDAR NR1, NR2A and NR2B subunit mRNA expression was examined by *in situ* hybridisation histochemistry in coronal sections from vehicle and quinolinic acid treated rats. Representative autoradiographs showing the distribution and intensity of NR1, NR2A and NR2B NMDAR subunit mRNA expression are shown in figures 5.11.

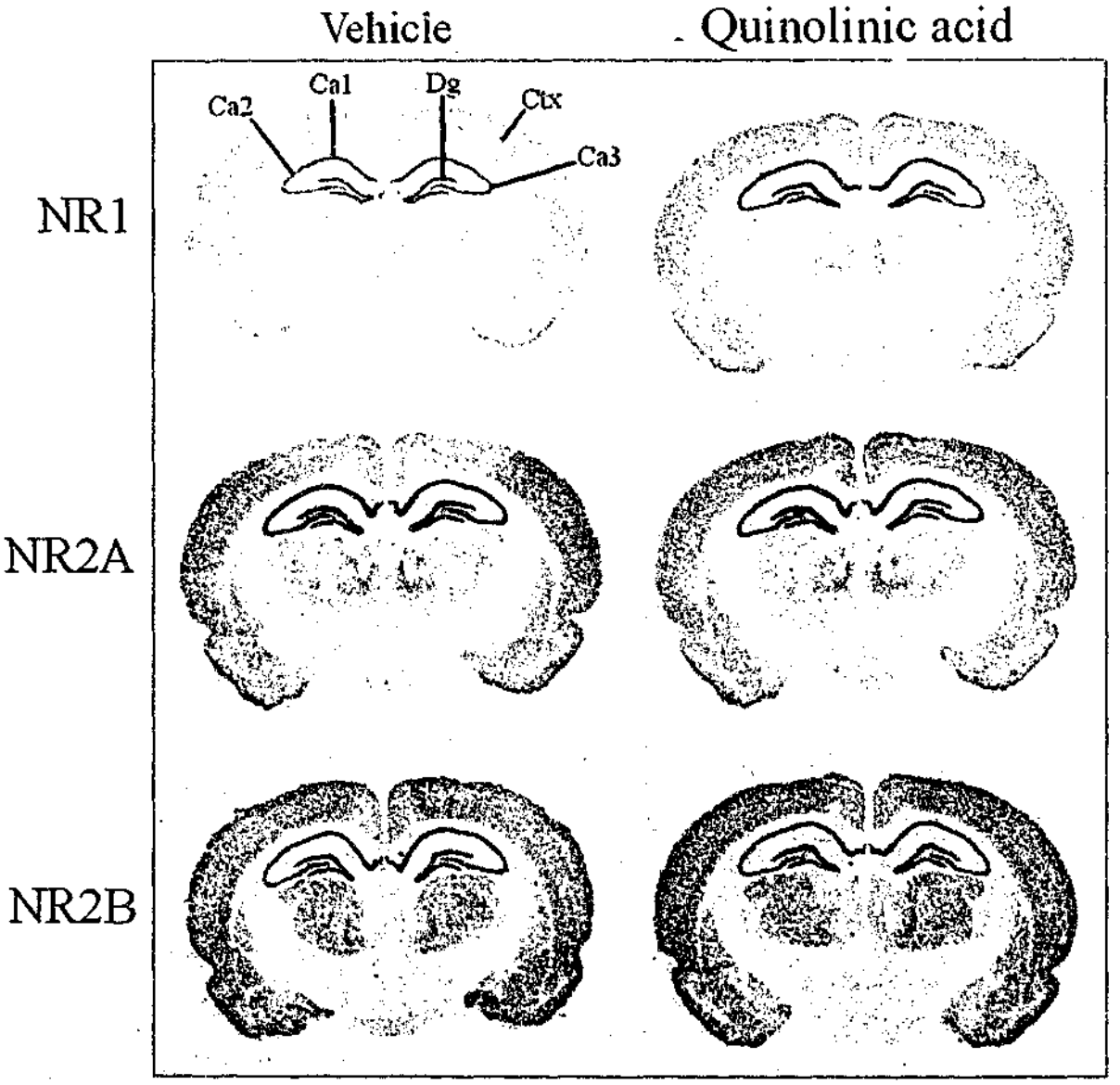


Figure 5.11. NMDAR subunit mRNA expression in representative coronal sections approximately 3.6 mm caudal to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus. Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus.

Rats treated with an injection of quinolinic acid into the MBN exhibited a significant increase in the level of NR1 and NR2B NMDAR expression relative to rats treated with vehicle (fig 5.12). Specifically, quinolinic acid treated rats exhibited an increase in NR1 mRNA expression of 25 – 43% in the hippocampus and 61% in the cortex respectively, and NR2B levels were significantly up-regulated by 21% in the cortex but not affected in the hippocampal regions quantified. No significant change in NR2A mRNA expression was observed in any of the hippocampal or cortical regions tested.

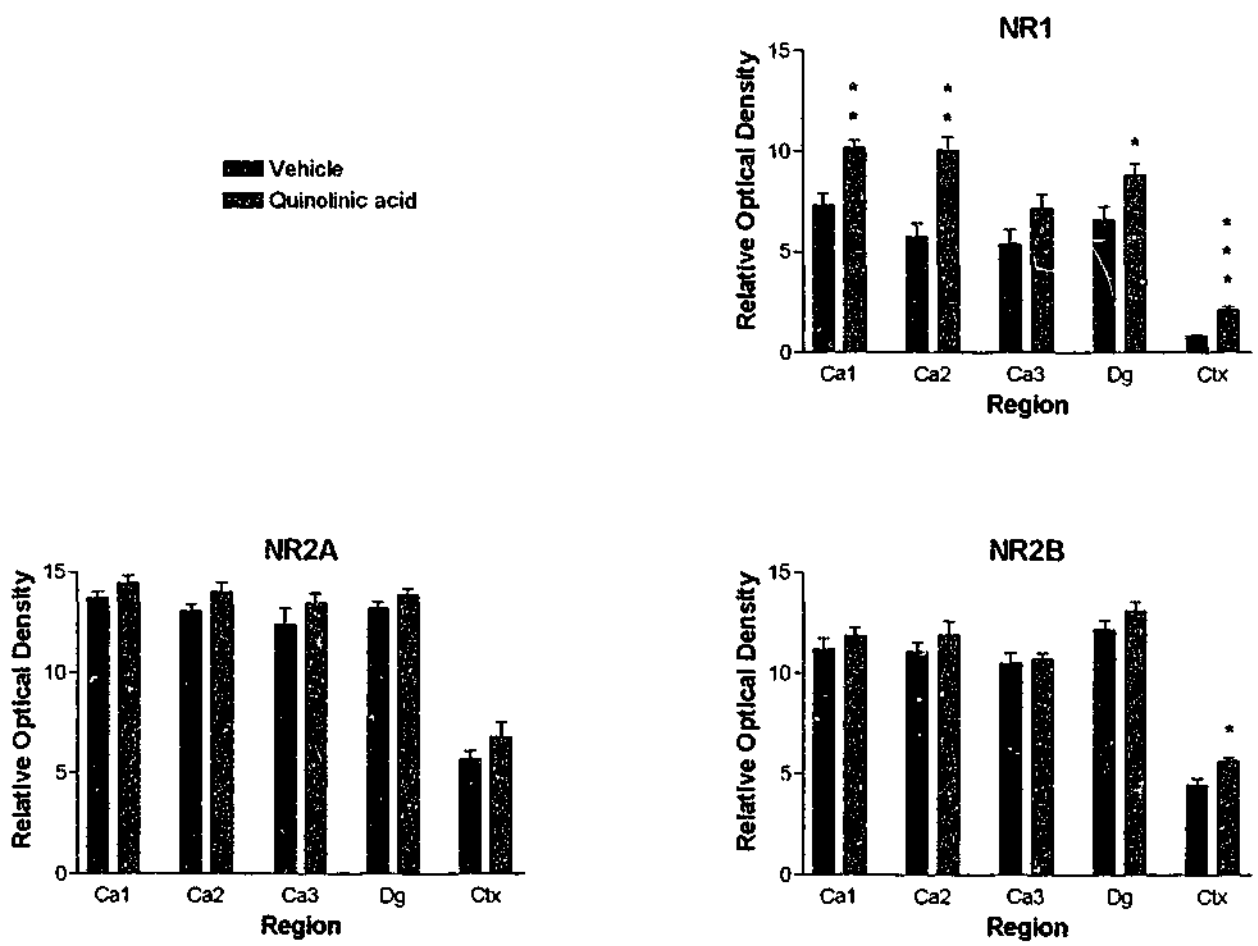


Figure 5.12. Levels of NR1, NR2A and NR2B NMDAR subunit mRNA expression in selected regions of coronal sections approximately 3.6 mm caudal to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus, quantified in terms of relative optical density (ROD). Abbreviations: Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus. The mean ROD level in the quinolinic acid treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

5.3.6 APP695, BACE, PS1, PS2 AND TACE mRNA EXPRESSION

The expression of APP695 and four proteins (BACE, PS1, PS2 and TACE) that play critical roles in the proteolytic processing of APP was examined in coronal sections from vehicle and quinolinic acid treated rats using *in situ* hybridisation histochemistry. Representative sections showing the distribution and intensity of the expression of these mRNAs in both vehicle and quinolinic acid treated groups are shown in figure 5.13.

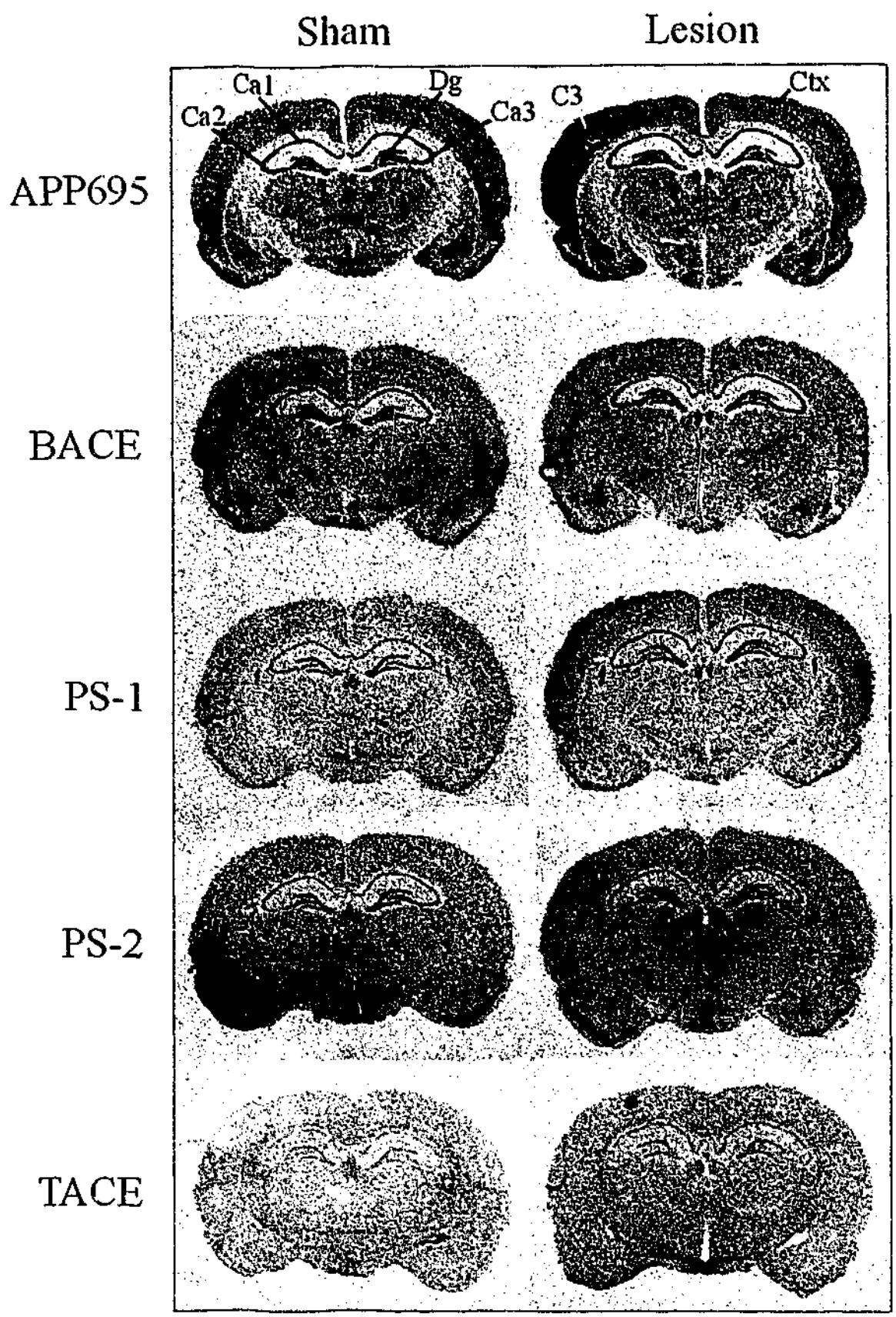


Figure 5.13. APP695, β-site APP-cleaving enzyme (BACE), presenilin-1 (PS1), presenilin-2 (PS2) and tumour necrosis factor-α-converting enzyme (TACE) subunit mRNA expression in representative coronal sections approximately 3.6 mm caudal to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus. Abbreviations: Ctx, cortex; C3, cortical layer 3; Dg, dentate gyrus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Excitotoxic nbM lesions, using quinolinic acid, caused a significant increase in APP695, TACE, PS1 and PS2 but not BACE mRNA expression in a number of

hippocampal and cortical regions (5.14). APP mRNA expression was increased by 10 – 28 % in the Ca1 and Ca2 fields of the hippocampus and in the cortex. TACE and PS1 mRNA expression was increased by 22 – 59 % and 23 – 40 % respectively throughout the hippocampus and cortex. The expression of PS2 mRNA was increased in the cortex by 26 % but was not significantly effected in the hippocampus. No significant change in the level of BACE mRNA expression was seen in any of the cortical or hippocampal regions examined in this study.

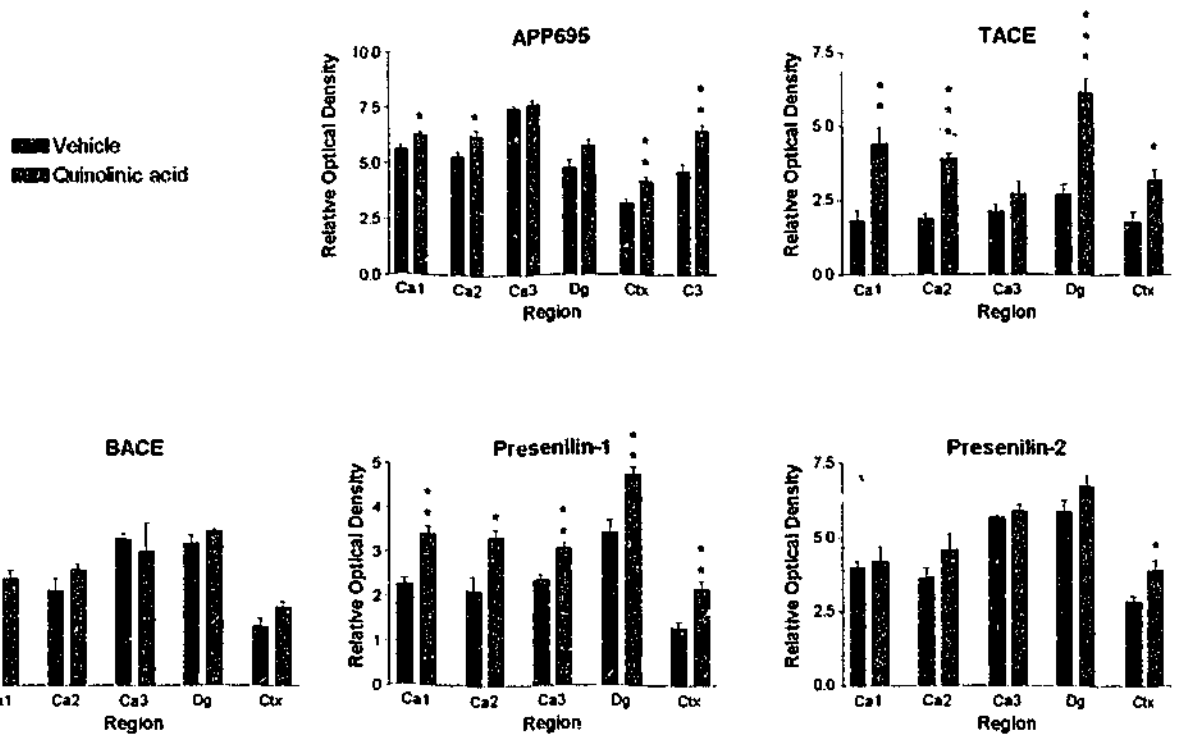


Figure 5.14. Levels of APP695, β-site APP-cleaving enzyme (BACE), presenilin-1, presenilin-2 and tumour necrosis factor-α-converting enzyme (TACE) mRNA expression in selected regions of coronal sections approximately 3.6 mm caudal to bregma from rats 7 days after injection of either vehicle or quinolinic acid into the magnocellular basal nucleus, quantified in terms of relative optical density (ROD). Abbreviations: Ctx, cortex; C3, cortical layer 3; Dg, dentate gyrus. The mean ROD level in the quinolinic acid treated groups was compared to the mean level of ROD in the corresponding vehicle group using an unpaired student's t test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

5.4 DISCUSSION

The significant reduction in cortical but not hippocampal AChE levels seen 7 days after excitotoxic lesioning of the MBN is consistent with the topographical organization of the cholinergic projections originating in this basal forebrain region (Mesulam et al., 1983; Rye et al., 1984). The magnitude of this reduction, 26 – 32%, is similar to the 28% reduction previously reported by Gutierrez et al., (1999). Notably, this response was fairly modest compared to the 74 – 80% loss of cortical AChE levels reported in the previous chapter as a result of selective cholinergic lesioning using 192-IgG-saporin. It is interesting then that, generally speaking, the excitotoxic lesions rather than 192-IgG-saporin treatment produced greater changes in the various neurochemical markers examined.

The increase in the expression of α_4 and β_2 nAChR subunit mRNA described here has also been reported by both Miyai et al., (1990) and Burghaus et al., (2001) in response to excitotoxic basal forebrain lesions. Interestingly, both of these studies described this as being a transient effect with the increase in nAChR subunit expression peaking at one week after excitotoxic lesions but returning to control levels 4 weeks after the lesion. Although it seems reasonable to hypothesise that the transient increase in these two nAChR subunits may underlie an up-regulation of the $\alpha_4\beta_2$ nAChR subtype, surprisingly it is reported here that there was no significant change in [^{3}H]epibatidine binding under the same conditions. This is consistent with previous findings which report no change in either [^{3}H]nicotine (Miyai et al., 1990) or [^{3}H]epibatidine (Bednar et al., 1998) binding following excitotoxic basal forebrain lesions. As discussed by Miyai et al., (1990), it is possible that there was in fact an increase in nAChRs on cortical neurons but a loss of presynaptic nAChRs on basal forebrain afferents may mask this effect. i.e. in radioligand binding experiments the net result of an increase in nAChRs on one population of cortical neurons

and a decrease in another population, may be no detectable change in the overall nAChR level. Because *in situ* hybridisation histochemistry detects mRNA at the level of the cell body, the nAChR subunit mRNAs detected in the cortex necessarily represent expression in intrinsic cortical cell bodies and are not representative of nAChRs located presynaptically on neuronal afferents which originate in other regions. Radioligand binding studies, however, do not distinguish between nAChRs located on these two neuronal populations. Therefore the increase of both α_4 and β_2 subunit mRNA and lack of change in [^{3}H]epibatidine binding in the cortex following MBN injections of quinolinic acid, may reflect an up-regulation of $\alpha_4\beta_2$ nAChRs on intrinsic cortical neurons and a corresponding loss of presynaptic $\alpha_4\beta_2$ receptors from cortically projecting basal forebrain afferents. Evidence has been published from two separate studies which support the idea that there is indeed an upregulation of nAChRs on cortical neurons (Abdulla et al., 1995) and a loss of presynaptic nAChRs on basal forebrain afferents projecting to the cortex (Bednar et al., 1998) following excitotoxic lesioning of the basal forebrain. Abdulla et al., (1995) have reported an increase in the sensitivity of cortical neurons 2 weeks after excitotoxic MBN lesions with the proportion of neurons responding to iontophoretically applied nicotine increasing from 32% to 54% following the lesion. Although this result is indicative of an increase in cortical nAChR density following MBN lesions, it was reported in the same study that there was also a significant reduction in cortical levels of [^{3}H]nicotine binding. Like Miyai *et al* (1990), Abdulla et al., (1995) have also argued that an increase in the expression of nAChRs on intrinsic neurons coupled with a significant loss of presynaptic nAChRs on cortically projecting basal forebrain afferents may explain their results. A study by Bednar et al., (1998) has similarly reported a significant reduction in cortical [^{3}H]cytisine binding 5 weeks after excitotoxic MBN lesions and have interpreted this result as a loss of cortical nAChRs located presynaptically on basal forebrain projections. Detection of this net reduction in cortical nAChR binding would not be compromised by

an up-regulation of nAChRs located postsynaptically relative to basal forebrain projections because, according to both Miyai et al., (1990) and Burghaus et al., (2001), there is no longer a discernable up-regulation of cortical nAChR subunit mRNA at this time point. Interestingly, Bednar et al., (1998) also report that selective cholinergic lesions with 192-IgG-saporin did not alter [^{3}H]cytisine binding levels 5 weeks after treatment and, taken together with the results showing a reduction in [^{3}H]cytisine binding 5 weeks after excitotoxic lesioning, have interpreted this result as indicating that most of the presynaptic basal forebrain nAChRs lie on non-cholinergic rather than cholinergic basal forebrain projections.

In addition to the significant increase in α_4 and β_2 mRNA in cortical regions reported here, there was also a significant up-regulation of β_2 mRNA in the hippocampal regions examined. Although MBN neurons do not project extensively to the hippocampus, there is extensive communication between hippocampal neurons and cortical neurons, particularly those that reside in the entorhinal cortex. Therefore the disruption of basal forebrain transmission to certain cortical neurons as a result of MBN lesions may, subsequently, affect communication between these cortical neurons and hippocampal neurons. This may be the basis for changes in neurochemical markers in the hippocampus, such as β_2 nAChR mRNA following excitotoxic MBN lesions. There may also be some significant level of innervation of the hippocampus by non-cholinergic neurons originating in the MBN. While it has been demonstrated that cholinergic MBN neurons project predominately to neocortical areas (Mesulam et al., 1983; Rye et al., 1984), less is known about the topographic organization of ascending non-cholinergic MBN projections. The increase in β_2 mRNA expression throughout the brain may represent an increase in the expression of nAChR subtypes other than the major $\alpha_4\beta_2$ isoform. Particularly in hippocampal regions where no detectable levels of α_4 mRNA signal was observed. Transfection studies using *xenopus* oocytes have demonstrated that in addition to the $\alpha_4\beta_2$

subtype, the β_2 subunit also forms functional and pharmacologically distinct nAChR subtypes when constructed as $\alpha_2\beta_2$, $\alpha_3\beta_2$ and $\alpha_3\beta_2\alpha_5$ ionophores. Unfortunately, there are currently no selective radioligands available to detect these nAChR subtypes. One of the advantages of measuring individual nAChR subunits at the mRNA level is that it is possible to predict potential changes in nAChR subtypes for which there are no selective radioligands.

In addition to the increase in hippocampal β_2 subunit expression, there was also an increase in the expression of α_7 subunit mRNA in the Ca1 and Ca2 fields of the hippocampus as a result of excitotoxic MBN lesioning. Notably, there was also a significant increase in [^{125}I]α-BGT binding to α_7 nAChRs in the same regions. Therefore it would be reasonable to conclude that there is an upregulation of α_7 nAChRs on hippocampal neurons in the Ca1 and Ca2 fields in response to excitotoxic MBN lesioning and that this response is likely to mediated at the mRNA level. So far as can be determined, there has been no information previously published describing the affect of excitotoxic basal forebrain lesions on the expression of α_7 subunit mRNA or [^{125}I]α-BGT binding. Speculatively, according to the hypothesis offered by both Miyai *et al* (1990) and Abdulla et al., (1995), which describes the level of radioligand binding to nAChRs following MBN lesions as being dependent on the net effect between an loss of presynaptic nAChRs on basal forebrain projections and an upregulation of nAChRs on intrinsic neurons in the projected target areas; the overall upregulation of [^{125}I]α-BGT binding reported here may reflect a lack of significant levels of α_7 nAChRs located presynaptically on basal forebrain afferents.

The increase in cortical and hippocampal nAChR subunit mRNA expression and [^{125}I]α-BGT binding in response to excitotoxic MBN lesions may represent a homeostatic response to a loss of basal forebrain driven neurotransmission in these areas. A study by Abdulla et al., (1994) has demonstrated a significant reduction in the firing rate of cortical

neurons following MBN lesions. A significant proportion of the neurons modulated by basal forebrain activity are likely to be glutamatergic (Flint et al., 1985; Lamour et al., 1982; Reine et al., 1992). In support of this, there is a significant depression of the spontaneous release of glutamate from cortical slices of rats that have received excitotoxic basal MBN lesions (Szerb et al., 1990). Reine et al., (1992) have reported a significant reduction in the Km value for glutamate uptake in both the cortex and hippocampus following excitotoxic MBN lesions. Reductions in the Km of high affinity glutamate transport has previously been correlated with a corresponding reduction in glutamatergic neuronal activity (Nieoullon et al., 1983). As has been reported here, Reine et al., (1992) also report that the lack of MBN projections to the hippocampus is reflected by a lack of change in hippocampal cholinergic activity following MBN lesions and have suggested that the reduction in glutamate uptake in this region may result from changes in perforant path transmission. The perforant path, which originates in the entorhinal cortex, is the major source of glutamatergic input to the hippocampus and also receives major inputs from the amygdaloid nuclei and various cortical areas that all share connections with the MBN (Beckstead, 1978; Koliatsos et al., 1988; Van Hoesen et al., 1975; Woolf et al., 1982). As discussed in the previous chapter, activation of the α_7 nAChR subtype has been shown to facilitate glutamatergic transmission in the Ca1 field of the hippocampus (Gray et al., 1996). Therefore, the up-regulation of α_7 type nAChRs in this region following MBN lesions may represent a homeostatic response to a reduced level of glutamatergic transmission in this area resulting from changes in the level of stimulation of glutamatergic Ca1 neurons by perforant path input. In an analogous manner, the up-regulation of α_4 and β_2 nAChR mRNA may be a homeostatic response to a depressed level of activity of other neuronal populations which are modulated by nAChR subtypes incorporating these subunits. For instance, the $\alpha_4\beta_2$ subtype is thought to positively modulate GABAergic transmission (Alkondon et al., 1997b; Lu et al., 1998) and interestingly Abdulla et al.,

(1994) have reported that there is a significant increase in cortical GABAergic tone following excitotoxic lesioning of the MBN. Speculatively, this may be the result of stimulation of an increased number of $\alpha_4\beta_2$ nAChRs on cortical GABAergic interneurons by the remaining cholinergic basal forebrain projections. A useful direction for further work in this area may involve examining the effect of selective nAChR ligands on the release of different neurotransmitters in the cortex and hippocampus following basal forebrain lesions.

The significant increase in the expression of hippocampal and cortical NMDAR subunit mRNA reported here following excitotoxic MBN lesions is also consistent with the idea that glutamatergic neurons in this region may up-regulate their receptors as a homeostatic response to a depression in the activity of these neurons caused by a loss of neuronal input from the basal forebrain. There was a significant increase in the level of NR1 mRNA throughout the hippocampus and cortex and NR2B mRNA in the cortex of MBN lesioned rats 7 days after the lesion. No significant change was seen in the expression of NR2A mRNA in these regions. While the increase in cortical NR1 and NR2B mRNA may represent the up-regulation of a specific NMDAR subtype incorporating these subunits, the up-regulation of NR1 expression in the hippocampus may represent a change in other NMDAR populations that do not incorporate the NR2A or NR2B subunits. Notably, while an increase in NR1 and NR2B mRNA has been reported here as a result of excitotoxic basal forebrain lesions, in the previous chapter it was reported that selective cholinergic basal forebrain lesioning results in an up-regulation of NR2A NMDAR subunit mRNA. Collectively, these results suggest that cholinergic and non-cholinergic basal forebrain projections are likely to affect different populations of NMDAR subtypes in telencephic regions. While this study shows that telencephic NMDAR subunit mRNA levels are regulated by basal forebrain activity, a useful expansion of this work would involve binding studies using selective NMDAR ligands to

correlate the changes in subunit mRNA expression with changes in specific NMDAR subtypes.

When compared to the results regarding 192-IgG-saporin treatment described in the previous chapter, excitotoxic lesioning of basal forebrain nuclei was associated with substantially less damage to cholinergic neurons but produced greater changes in the NMDA and nicotinic markers examined. It is therefore concluded that non-cholinergic basal forebrain neurons may play an important role in regulating NMDA and nicotinic subunit mRNA expression and receptor levels. As discussed in the introduction, non-cholinergic MBN neurons are predominately GABAergic and synapse on local GABAergic interneurons in the cortex that provide inhibitory control of other cortical neurons, a large percentage of which are glutamatergic. A loss of GABAergic MBN neurons may therefore be expected to decrease the activity of cortical glutamatergic neuronal activity as a result of increased cortical GABAergic tone. Considering the importance of cortical and hippocampal glutamatergic transmission in memory function, a loss of glutamatergic activity may underlie the cognitive impairments associated with basal forebrain lesions. The finding that the degree of cholinergic basal forebrain dysfunction does not correlate well with the degree of cognitive impairment following excitotoxic basal forebrain lesions (Dunnett et al., 1987; Robbins et al., 1989a; Robbins et al., 1989b) raises the possibility that non-cholinergic basal forebrain neurons may play a more important role in mediating this effect. Similarly, the work presented in this and the previous chapter collectively indicate that non-cholinergic rather then cholinergic basal forebrain neurons may play an important role in regulating NMDAR and nAChR markers in basal forebrain target areas.

In summary, this work has demonstrated that a loss of basal forebrain activity results in an up-regulation of NMDAR and nAChR subunit mRNAs and [^{125}I]α-BGT binding, and evidence has been detailed which implicates non-cholinergic MBN neurons as

mediating this effect. Initially, the relevance of these results to the pathological changes seen in AD may seem questionable considering that studies using post-mortem AD tissue have demonstrated a significant *decrease* in NMDAR and nAChR markers and little data has been published from these studies describing the degeneration of non-cholinergic basal forebrain neurons. However, the use of relatively unspecific neuronal markers, such as GAD, for the detection of GABAergic basal forebrain neurons may prevent the accurate quantification of this neuronal population in many studies. Furthermore, as discussed in detail in the previous chapter, the widespread loss of many neurochemical markers, such as nAChRs and NMDARs, in post-mortem AD studies may well reflect the non-specific degeneration of many neuronal populations associated with end-stages of the disease. The up-regulation of hippocampal and cortical nAChR and NMDAR markers described here might be an important change associated with the degeneration of the basal forebrain structure at earlier stages of AD. Importantly, it has been reported here that there is an up-regulation of specific neurochemical markers in the hippocampus and cortex in an experimental paradigm that has consistently been shown to impair certain aspects of memory and cognition. This may represent a homeostatic response in an attempt to compensate for the neurochemical imbalances which cause cognitive dysfunction and therefore these neurochemical markers (nAChRs and NMDARs) may be important therapeutic targets for the cognitive dysfunction associated with AD.

In addition to an up-regulation of nicotinic and NMDA receptor markers, it has also been reported here that excitotoxic basal forebrain lesions cause a significant increase in the mRNA expression of APP695 as well as a number of proteins involved in its proteolytic processing, including PS1, PS2 and TACE. The significant increase in cortical and hippocampal APP mRNA expression seen 7 days after quinolinic acid injection into the nbM is consistent with a number of studies which have also reported increases in APP at both the mRNA (Wallace et al., 1993; Wallace et al., 1991) and protein (Harkany et al.,

2000a; Iverfeldt et al., 1993) level in response to excitotoxic basal forebrain lesions. In light of the results reported in the previous chapter showing no change in APP mRNA levels 7 days after 192-IgG-saporin treatment, it is unlikely that the up-regulation in cortical and hippocampal APP following excitotoxic lesions is related specifically to a loss of cholinergic innervation of these areas. This raises the interesting possibility that GABAergic basal forebrain activity may play a role in regulating APP transcription. A study by (Wallace et al., 1993) has reported that increased cortical APP expression following the loss of basal forebrain neurons is likely to be related specifically to reduced neuronal activity rather than due to non-specific neuronal damage and the physical loss of neurons. It was demonstrated in this study that the up-regulation of cortical APP mRNA levels associated with injections of excitotoxic basal forebrain lesions can be simulated by injecting the lidocaine into the nbM. Lidocaine is a sodium channel antagonist that reversibly disrupts neuronal transmission without causing neuronal damage. After showing that APP transcription is reversibly up-regulated following injection of lidocaine into the MBN, Wallace et al., (1993) concluded that APP levels in the cortex may be regulated by functional neuronal innervation from the basal forebrain. The results presented here, as well as in the previous chapter, indicate that non-cholinergic basal forebrain neurons may play an important role in this context. It has also been reported here that excitotoxic lesioning of the basal forebrain results in a significant up-regulation of PS1, PS2 and TACE mRNA in the cortex and hippocampus. Based on a considerable amount of evidence implicating these proteins in the proteolytic processing of APP (for review see Nunan et al., 2000), this result is consistent with the finding that there is an increase in the production of sAPP fragments in response to neuronal degeneration (Iverfeldt et al., 1993). Iverfeldt et al., (1993) has reported that after quinolinic acid induced lesions of the striatum there is a significant increase in the level of sAPP fragments and concomitant decrease in full length APP695 in both the lesioned area and in those projection areas where the axons

of the lesioned neurons terminate. It was concluded that proteolytic processing of APP is increased in response to neuronal degeneration. The results presented here indicate that this effect may be mediated by an up-regulation in the expression of various proteins that play impotant roles in APP processing. As detailed in the previous chapter, 192-IgG-saporin induced cholinergic lesioning of the basal forebrain did not significantly affect PS1, PS2 or TACE mRNA levels. Therefore the finding that there is an up-regulation of these mRNAs following excitotoxic lesions suggests that non-cholinergic basal forebrain neurons may play a role in mediating this effect. It is also possible that a greater degree of neuronal damage associated with excitotoxic, as apposed to immunotoxic, lesioning is a factor in up-regulating PS1, PS2 and TACE mRNA. Relative to 192-IgG-saporin treatment, the non-specific damage to a greater subset of neuronal cells caused by quinolinic acid injection may reasonably be expected to be associated with a greater gliotic response to neuronal degeneration. While 192-IgG-saporin injection does not produce local gliosis in excess of that associated with vehicle injection (Holley et al., 1994), significant microglial and macrophage invasion appears to represent an early stage in excitotoxic lesion induced gliosis (Marty et al., 1991). Activated microglial cells release interleukin-1 (Giulian et al., 1986), which acts as a mitogen for astrocytes. Notably, interleukin-1 has been shown to induce the secretion of sAPP fragments (Buxbaum et al., 1992). Collectively, the results reported here and in the previous chapter suggest that the degree and mechanism of neuronal damage may be important factors that regulate APP processing, or at least the expression of certain proteins involved. Interestingly, while there was an increase in PS1, PS2, and TACE mRNA following the excitotoxic nbM lesions, there was no change in the level of BACE mRNA. This pattern of changes is consistent with a specific up-regulation in proteins involved in the processing of APP along the non-amyloidogenic α-secretase pathway (refer to fig. 1.1, Chapter1, *General Introduction*). Processing along this pathway prohibits the formation of Aβ, by cleaving within the Aβ sequence, and results in the

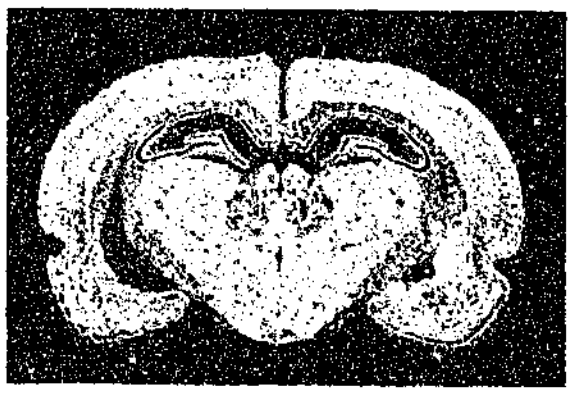
formation of sAPPα and p3. A number of studies have described potent neuroprotective and neurotrophic effects mediated by sAPPα. In particular, it is thought that sAPPα plays an important role in stabilising intracellular calcium levels and preventing damage associated with excessive neuronal stimulation (Li et al., 2000; Mattson et al., 1993b; Mattson et al., 1993a). The increase in PS1, PS2 and TACE levels reported here may reflect an up-regulation in sAPPα production in an attempt to counter the excessive neuronal stimulation and damage caused by quinolinic acid induced excitotoxicity. It is well established that a high-efficacy endogenous mechanism of neuroprotection in the brain is the enhanced release of neurotrophic/neuroprotective factors at the site of neuronal damage (Ciccarelli et al., 1999; Hortobagyi et al., 1998; Masliah et al., 1997; Ohsawa et al., 1999). While upregulation of APP processing along the non-amyloidogenic α-secretase pathway may represent a response to neuronal damage in the normal brain, a consequence of the pathology associated with AD may be up-regulation of the amyloidogenic β-secretase pathway in response to neurodegeneration. Of course this is certainly likely to be the case in familial forms of the disease, which by definition are associated with aberrant APP processing.

It has been demonstrated here and in the previous chapter that a loss of both cholinergic and non-cholinergic basal forebrain neurons may contribute to the up-regulation of APP in the cortex and hippocampus. Furthermore, there is also an up-regulation of proteins involved in the non-amyloidogenic processing of APP in response to excitotoxic neuronal damage. An up-regulation of these proteins coupled with an up-regulation of the APP substrate may be expected to significantly increase the formation of sAPP fragments. Clearly the type of APP processing proteins up-regulated will affect the type of sAPP fragments produced. The results presented here indicate that the normal response to excitotoxic damage is an up-regulation of APP processing along the α-secretase pathway and, consequently, the production of sAPPα and p3. It would be

interesting to investigate the possibility that, in AD brains, neuronal damage is associated with pathological up-regulation of amyloidogenic APP processing along the β-secretase pathway and the production of sAPPβ and Aβ. Circumstantially at least, this hypothesis is supported by the consistently reported finding that Aβ plaques are closely associated with dystrophic neurites (Dickson, 1997; Terry et al., 1994) in AD brains.

Chapter 6

Investigations into possible interactions between β-amyloid and the α_7 nAChR in vitro and in vivo



6.1 INTRODUCTION

As has been discussed, one of the greatest challenges in AD research involves the accurate characterisation of the neurodegenerative process based on a unifying model that describes the relevance of and relationship between the wide variety of neurochemical changes associated with the disease. The degeneration of the cholinergic basal forebrain system (Beach et al., 1997; Bigl et al., 1987; Bowen et al., 1976; Bowen et al., 1982; Davies et al., 1976; Perry et al., 1977; Zubenko et al., 1989), a loss of cortical and hippocampal nAChR density (Flynn et al., 1986; Guan et al., 2000; Nordberg et al., 1990; Nordberg et al., 1986; Sugaya et al., 1990; Warpman et al., 1995) and the accumulation of Aβ rich plaques in these areas (Alzheimer, 1907; Dickson, 1997; Glenner et al., 1984; Masters et al., 1985; Terry et al., 1981; Terry et al., 1970) are some of the most consistently reported features associated with AD. Consequently, a great deal of research has focussed on a possible relationship between Aβ deposition and cholinergic loss or dysfunction in AD. Establishing a causal relationship between these two major pathological changes in AD brains has been somewhat of an elusive goal in AD research and one pursued by proponents of the 'cholinergic hypothesis' (Bartus et al., 1982) and 'amyloid cascade hypothesis' (Hardy, 1997) of AD neuropathology alike. As discussed in detail in chapter 3, a compelling body of evidence has been documented over the last decade that directly implicates Aβ peptides in both the degeneration and modulation of cerebral cholinergic neurons (for review see Auld et al., 1998). Relatively little, however, is known about the specific nature of interaction between Aβ peptides and cholinergic neurons.

Two studies recently published by Wang et al., (2000a & 2000b) report that $A\beta_{(1-42)}$ binds selectively to the α_7 subtype of the neuronal nicotinic acetylcholine receptor (nAChR) with picomolar affinity. This finding is particularly interesting considering the

important role that α_7 nAChRs are thought to play in learning and memory (Fujii et al., 2001; Levin et al., 1999; Matsuyama et al., 2000; Meyer et al., 1997; Rezvani et al., 2001), and offers a possible explanation for the mechanism of interaction between Aβ peptides and cholinergic neurons. The α_7 nAChR subtype displays permeability to Ca^{2+} ions significantly higher than has been reported for other ligand gated ion channels, including the NMDA subtype of glutamate receptors (Seguela et al., 1993). The permeability ratio (P_{Ca}^{2+}:P_{Na}^{+}) of α_7 nAChRs is about 20, compared to 1.5 for other neuronal nAChRs and 0.2 for muscle nAChRs (Vernino et al., 1992). Such high permeability to Ca^{2+} may predispose the neuron following activation of these receptors to calcium overload neurotoxicity. Accordingly, Wang *et al* (2000 a,b) have hypothesized that the α_7 nAChR may mediate cytotoxicity through its interaction with $A\beta_{(1-42)}$ based on their findings that α_7 nAChRs and $A\beta_{(1-42)}$ co-localize in neuritic plaques and in AD neurons and that $A\beta_{(1-42)}$ binds selectively to α_7 nAChRs *in vitro* as determined in competition studies with α_7 nAChR specific ligands. Furthermore, they have indicated that the binding motif for $A\beta_{(1-42)}$ is likely to be contained within amino acids 12-28.

The aim of the work presented in this chapter has been to extend on the work described by Wang *et al* (2000 a,b) by investigating the regional distribution of $A\beta_{(1-42)}$ and $A\beta_{(12-28)}$ binding to α_7 nAChRs in areas selectively effected in AD pathology, such as hippocampus and cortex. This was achieved by examining competition of $A\beta_{(1-42)}$ and $A\beta_{(12-28)}$ with the selective α_7 nAChR ligand [^{125}I]α-BGT using autoradiographic techniques in coronal slices from rat brain. Two other α_7 nAChR selective ligands, choline and methyllcaconitite (MLA), were used in competition with [^{125}I]α-BGT to demonstrate that [^{125}I]α-BGT binding was displaceable in this preparation. Additionally, the effect of *in vivo* treatment with soluble $A\beta_{(1-40)}$ on [^{125}I]α-BGT binding to α_7 nAChRs as well as the expression of the α_7 nAChR subunit mRNA has been discussed.

6.2 METHODS

6.2.1 TREATMENT OF ANIMALS AND TISSUE PREPARATION

The following methods relating to animal treatment are based on those previously described in chapter 3, *3.2.1 Treatment of Animals and Tissue Preparation.*

Sprague-Dawley rats (250-300g) of either sex received continuous i.c.v. infusions for 14 days of either $A\beta_{(1-40)}$ peptide dissolved in vehicle consisting of 30% acetonitrile/0.1% trifluoroacetic acid (v/v) or vehicle alone. The animals were anaesthetised with pentobarbitone (60 mg/kg i.p.) and constrained to a tilted skull position (-0.3 mm) using a Kopf stereotaxic apparatus: A continuous i.c.v., 14 day infusion of either $A\beta_{(1-40)}$ (300 pmol/day) or vehicle was achieved using brain infusion kits (Alza corp., USA.) attached to modified miniosmotic pumps (pump rate of 1 µl/hr; Alza corp., USA). The osmotic minipumps were weighed before and after addition of the solution for i.c.v. infusion to ensure that they had been filled. After removing the plastic cap that covers the metal rod housed in the minipump a 4.5 cm length of polyethelene tubing was used to attach the minipumps to the infusion cannula. The minipumps were placed between the scapulae in a small subcutaneous cavity. A small hole was drilled into the skull and a capped infusion cannula was implanted into the left lateral ventricle at stereotaxic coordinates: 1.0 mm caudal to bregma, 1.5 mm left from the midline, 3.4 mm ventral from the cortical surface according to the atlas of Paxinos and Watson (1986). The infusion cannula was fixed in place by inserting a small stainless steel screw into the skull and then anchoring the cannula to the screw using dental cement.

Following treatment, animals were decapitated and the brains frozen over liquid nitrogen. A Reichert Jung cryostat was used to collect a series of 14 μm sections. Consecutive coronal sections were taken at 3.6 mm caudal to bregma and thaw mounted on poly-L-lysine coated slides. All tissue was stored at -70°C until required. A series of sections was also taken from untreated animals for use in the autoradiographic competition studies with $A\beta_{(1-42)}$ and [^{125}I]α-BGT.

6.2.2 [^{125}I]α-BUNGAROTOXIN BINDING TO MEMBRANES

The protocol for membrane binding is based on that described by Wang et al., (2000b). Freshly extracted hippocampal tissue from Sprague Dawley rats (250 – 300 g) of either sex was homogenised in 50 volumes (w/v) of ice-cold Na^+-HEPES using a glass homogeniser, and the homogenate was centrifuged at 42 000 g at 4°C for 10 min. The supernatant was discarded and the pellet was washed by resuspending it in 40 volumes (w/v) of ice-cold Na^+-HEPES and centrifuging at 42 000 g at 4°C for 10 min. This washing step was repeated three times before the pellet was finally resuspended in 25 volumes (w/v) of a buffered incubation medium containing Na^+-HEPES (10 mM, pH 7.4), 5 mM $MgCl_2$, 0.01% bovine serum albumin and 100 mM NaCl.

Each sample tube, tested in duplicate, contained a total volume of 1 ml including: 0.25 ml of the membrane suspension; 0.4 ml of incubation medium; 0.25 ml of [^{125}I]α-BGT in incubation medium (final concentration of 2 nM); 0.1 ml of the unlabelled competitor in dH_20. The competitors were pre-incubated with the tissue and incubation medium at 37°C for 20 min prior to the addition of [^{125}I]α-BGT. Non-specific binding was determined in the presence of 100 mM (–)-nicotine. The reaction proceeded for 1 h at 37°C and was terminated by vacuum filtration using a Brandell™ cell harvester and

washed twice with ice-cold Na^+-HEPES (10 mM, pH 7.4). The amount of bound [^{125}I]α-BGT was quantified by liquid scintillation spectrometry using a Packard Tri-Carb™ liquid scintillation spectrometer (counting efficiency 30-50%). Specific binding was calculated by subtracting non-specific binding from total binding and the percentage of specific [^{125}I]α-BGT binding was plotted against the concentration of unlabelled competitor used. Graphpad PRISM software (San Diego, USA) was used to fit displacement curves to the data and calculate IC_{50} values using non-linear regression analysis assuming one saturable binding site.

6.2.3 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY

The protocol for [^{125}I]α-BGT was based on that used by Whiteaker et al., (1999) and has been described in detail under Chapter 2, General Methodology, *2.3 [^{125}I]α-Bungarotoxin Autoradiography*. For the competition studies, the unlabeled competitors were included in the 20 minute pre-incubation step and maintained throughout the incubation with [^{125}I]α-BGT.

The autoradiographic images were digitally captured and [^{125}I]α-BGT binding in selected anatomical regions was quantified in terms of relative optical density using Scion imaging software (www.scioncorp.com) as previously described in general methods, *2.5 Quantitation*. Specific binding was calculated by subtracting non-specific binding from total binding.

For analysis of the competition studies, the percentage of specific [^{125}I]α-BGT binding was plotted against the concentration of unlabelled competitor used. Graphpad PRISM software (San Diego, USA) was used to fit displacement curves to the data and

calculate IC_{50} values using non-linear regression analysis assuming one saturable binding site. Each data point represents the average of measurements taken from 4 animals.

For analysis of the effects of *in vivo* $A\beta_{(1-40)}$ treatment, [^{125}I]α-BGT binding in coronal sections from $A\beta_{(1-40)}$ treated animals was compared to [^{125}I]α-BGT from vehicle treated animals using an unpaired student's t test. P values of less than 0.05 were taken to indicate statistical significance. Reported levels of [^{125}I]α-BGT binding represents the average of measurements taken from 4 animals.

6.2.4 In Situ Hybridisation Histochemistry

The protocol for in situ hybridisation is based on the methods described Wisden et al., (1994) and adapted by Loiacono et al., (1999), and has been described in detail under Chapter 2, General Methodology, *2.1 In Situ Hybridisation Histochemistry*.

6.3 RESULTS

6.3.1 [^{125}I]α-BUNGAROTOXIN BINDING TO MEMBRANES

The ability of $A\beta_{(1-42)}$, $A\beta_{(1-28)}$ or the α_7 nAChR selective ligand, choline, to bind to the α_7 nAChR subtype was determined by assessing the ability of these ligands to compete with [^{125}I]α-BGT binding in a preparation of hippocampal membrane fragments. [^{125}I]α-BGT was not displaceable by either $A\beta_{(1-42)}$ or $A\beta_{(1-28)}$ up to a concentration of 30 nM (fig. 6.0). Choline displaced [^{125}I]α-BGT binding with an IC_{50} (95% confidence limits) of 45.12 (28.30 – 71.93) mM. Non-specific binding, as defined by 100 mM (–)-nicotine, typically accounted for approximately 20-25% of the total signal (data not shown) and was subtracted from total binding when plotting the competition curves.

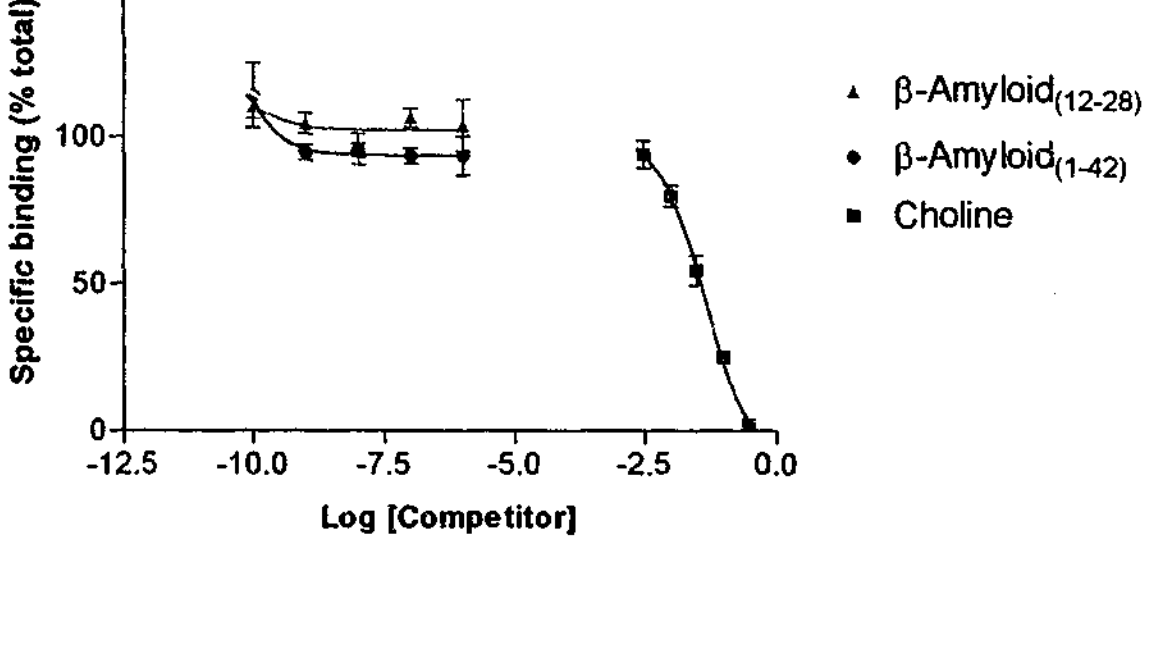


Figure 6.0. Competition curves for the displacement of [^{125}I]α-BGT (2 nM) by $A\beta_{(12-28)}$, $A\beta_{(1-42)}$ and choline in rat hippocampal membrane fragments. Graphpad PRISM software was used to fit displacement curves to the data and calculate IC_{50} values using non-linear regression analysis assuming one saturable binding site. Each data point represents the mean ± S.E.M. of measurements taken from four animals.

6.3.2 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY

[^{125}I]α-BGT binding in coronal slices approximately –3.6 mm caudal to bregma was used to assess the density and regional distribution of the α_7 nAChR subtype in the hippocampus and cortex (fig. 6.1). The ability of $A\beta_{(1-42)}$, $A\beta_{(1-28)}$ or the α_7 nAChR selective ligands, choline and MLA, to bind to α_7 nAChRs in selected anatomical regions was determined using computer assisted densitometry to assess the ability of these ligands to compete with [^{125}I]α-BGT binding in the whole slice preparation. Non-specific binding, as defined by 10 mM (–)-nicotine, ranged from <10% in the granular layer of the dentate gyrus to approximately 40% in the stratum radiatum area of the hippocampus (data not shown). Non-specific binding was subtracted from the total signal during quantitation. The use of MLA was employed in this paradigm, in addition to choline, based on the ability of MLA to displace [^{125}I]α-BGT binding (Whiteaker et al., 1999) at concentrations similar to those previously reported for Aβ peptides (Wang et al., 2000a & 2000b). MLA was not used in the homogenate competition protocol based on limited availability and the relatively higher amounts of MLA required as compared to the autoradiographic protocol.

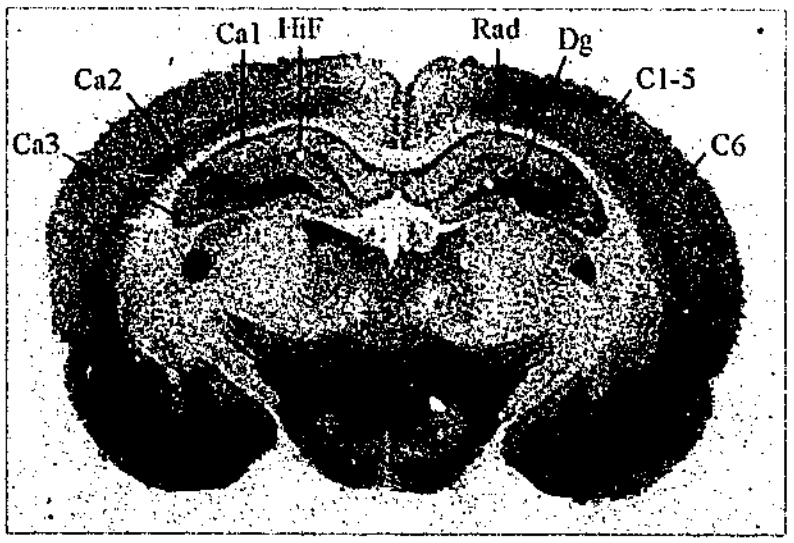


Figure 6.2. Total [^{125}I]α-BGT binding in a representative section approximately 3.6mm caudal to bregma. Abbreviations: Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Binding of [^{125}I]α-BGT to α_7 nAChRs was not displacable by either $A\beta_{(1-42)}$ or $A\beta_{(1-28)}$ up to a concentration of 30 nM in any of the regions examined (fig 6.3). In contrast, both MLA and choline were capable of completely displacing [^{125}I]α-BGT binding under the same conditions. The IC_{50} values for MLA and choline in the various regions examined are presented in Table 6.0.

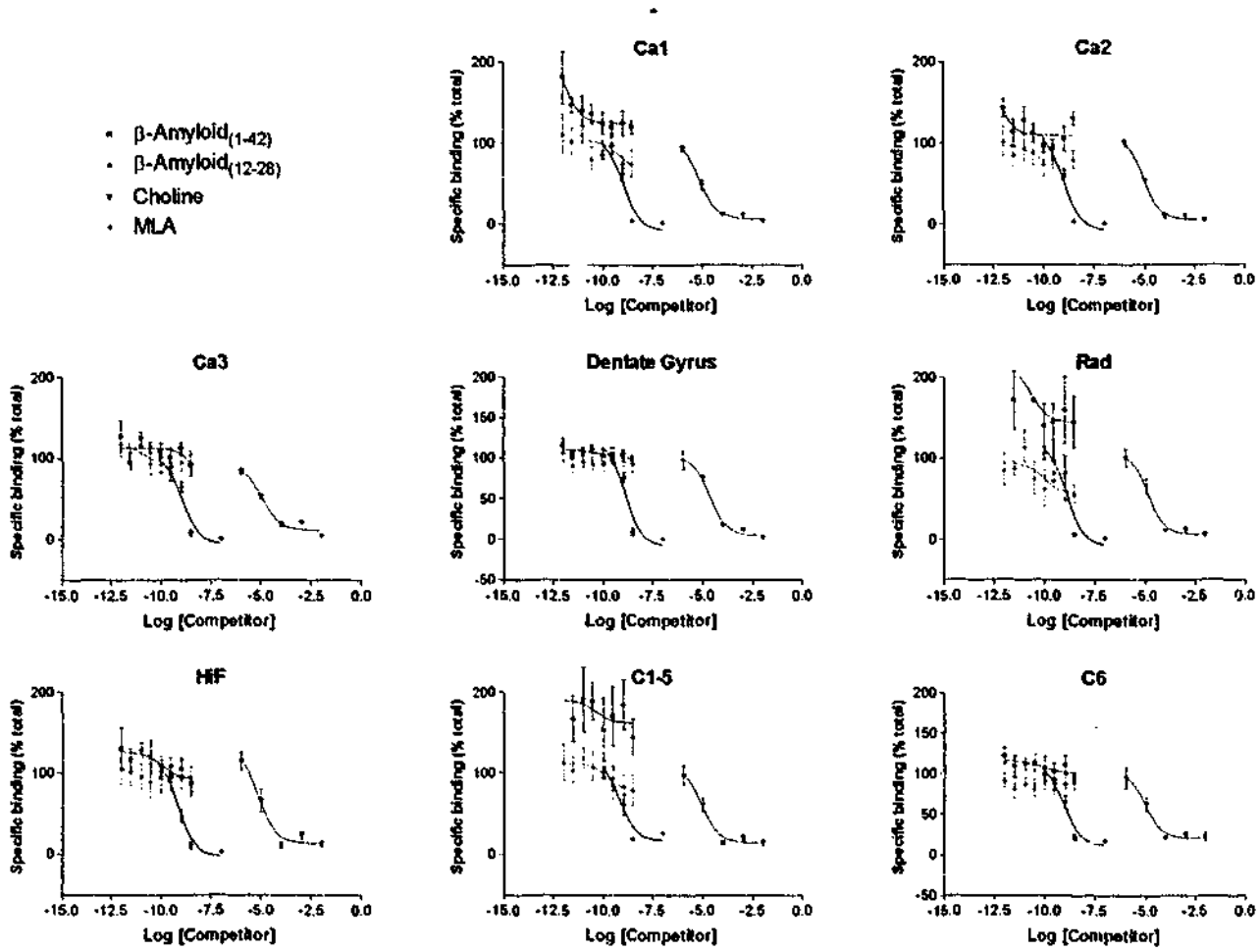


Figure 6.3. Competition curves for the displacement of [^{125}I]α-BGT (2 nM) by β-amyloid$_{(12-28)}$, β-amyloid$_{(1-42)}$, MLA and choline. Abbreviations: Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; HiF, hippocampal fissure; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). Graphpad PRISM software was used to fit displacement curves to the data and calculate IC_{50} values using non-linear regression analysis assuming one saturable binding site. Each data point represents the mean ± S.E.M. of measurements taken from four animals.

	IC_{50} (95 % CL)	
Region	**Choline (μM)**	**MLA (nM)**
Ca1	7.13 (4.49 – 11.33)	1.11 (0.87 – 3.20)
Ca2	7.86 (5.42 – 11.40)	0.96 (0.44 – 2.10)
Ca3	11.38 (5.90 – 21.96)	1.10 (0.53 – 2.29)
Dg	21.82 (10.73 – 44.37)	1.16 (0.51 – 2.63)
C1-5	8.91 (3.40 – 23.37)	0.62 (0.16 – 2.37)
C6	9.09 (3.13 – 26.43)	0.86 (0.42 – 1.76)
Rad	12.34 (5.56 – 27.35)	1.20 (0.28 – 5.19)
HiF	7.63 (2.75 – 21.17)	0.67 (0.25 – 1.81)

Table 6.0. IC_{50} values with 95% confidence limits for the displacement of [^{125}I]α-BGT binding in various telencephic regions in the rat brain by choline and MLA. Abbreviations: 95% CL, 95% confidence limits; Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). Graphpad PRISM software was used to calculate IC_{50} values using non-linear regression analysis assuming one saturable binding site.

6.3.3 [^{125}I]α-BGT Binding and α_7 Subunit Expression Following β-Amyloid$_{(1-40)}$ Infusions

The effect of *in vivo* treatment with $A\beta_{(1-40)}$ on the level of α_7 nAChR receptors as well as the expression of α_7 subunit mRNA was examined in a number of hippocampal and cortical regions, using *in situ* hybridisation histochemistry and [^{125}I]α-BGT autoradiography respectively. These results have also been reported in chapter 3, but are presented again here for clarity.

Continuous i.c.v. infusions of soluble $A\beta_{(1-40)}$ did not significantly effect the level of [^{125}I]α-BGT binding in any of the regions examined in this study (fig. 6.4; unpaired student's t test, $P > 0.05$).

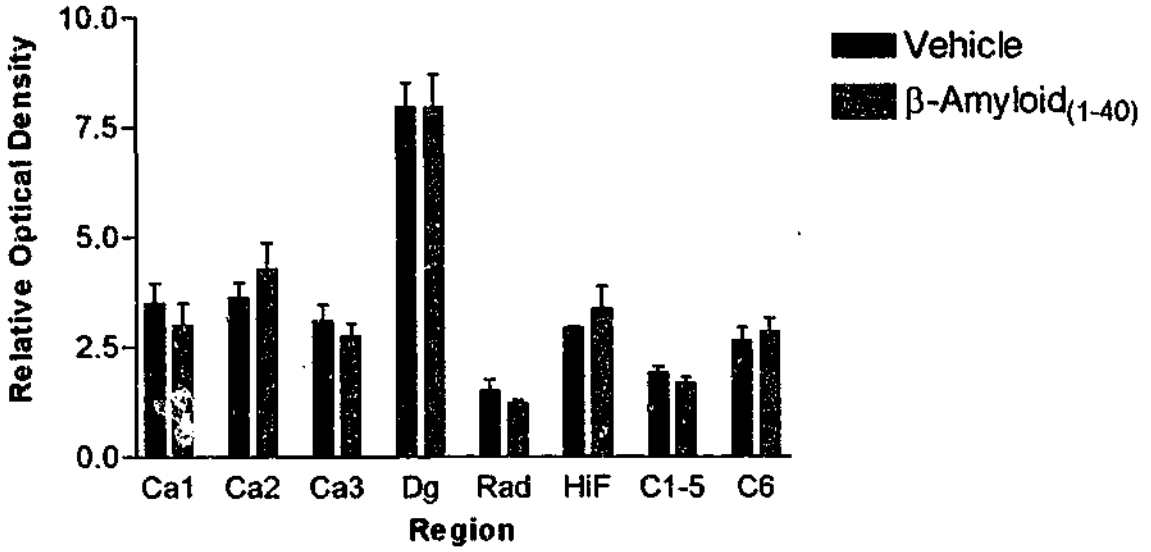


Figure 6.4. Levels of specific [^{125}I]α-BGT binding in selected regions of coronal sections approximately 3.6 mm caudal to bregma from rats following a continuous, 14 day i.c.v. infusion of either $A\beta_{(1-40)}$ or vehicle, quantified in terms of relative optical density (ROD). Abbreviations: C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD level in the $A\beta_{(1-40)}$ treated group was compared to the mean level of ROD in the vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

Although the expression of the α_7 nAChR subunit mRNA was consistently lower in $A\beta_{(1-40)}$ treated animals in the various regions examined, this was not a statistically significant effect (fig. 6.5; unpaired student's t test, $P > 0.05$).

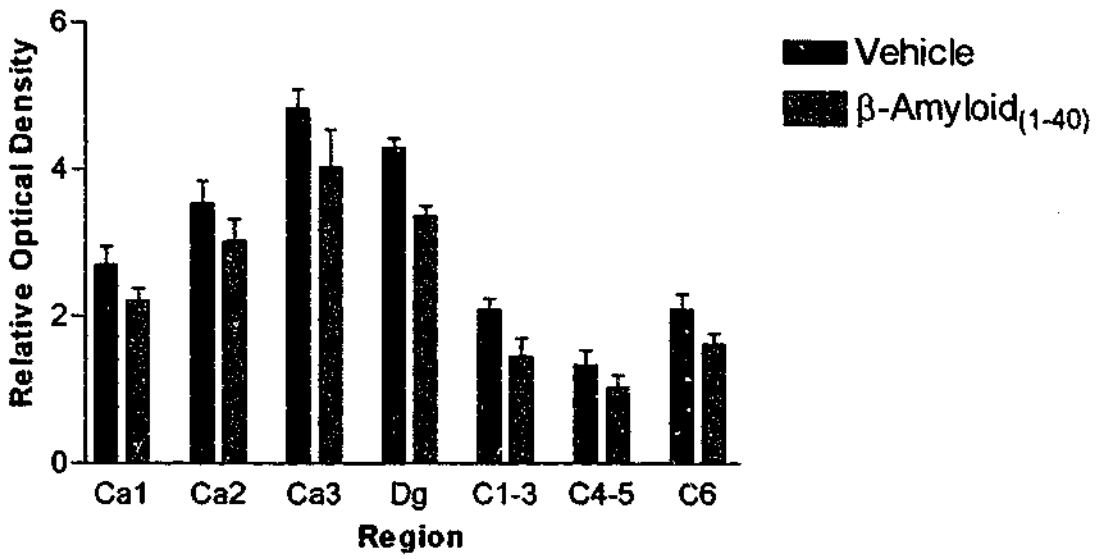


Figure 6.5. Levels of α_7 nAChR subunit expression in selected regions of coronal sections approximately 3.6 mm caudal to bregma from rats following a continuous, 14 day i.c.v. infusion of either $A\beta_{(1-40)}$ or vehicle, quantified in terms of relative optical density (ROD). Abbreviations: C1-3, cortical layers 1-3; C4-5, cortical layers 4-5; C6, cortical layer 6; Dg, dentate gyrus; ROD, relative optical density. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD level in the $A\beta_{(1-40)}$ treated group was compared to the mean level of ROD in the vehicle group using an unpaired student's t test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

6.4 DISCUSSION

While it is generally accepted that there is a loss of nAChRs associated with the progression of AD, there is some degree of inconsistency in the literature regarding how levels of the α_7 nAChR are specifically affected. Studies of post-mortem AD brain tissue have found either a reduced number (Guan et al., 2000) or no significant change (Martin-Ruiz et al., 1999; Sugaya et al., 1990) in the level of α_7 nAChR subunit protein relative to aged matched controls. An increase in α_7 subunit expression at the mRNA level in AD brains has also been reported (Hellstrom-Lindahl et al., 1999). Based on the results presented here, and those presented by Wang *et al* (2000a,b) there also seems to be some discordance in regard to whether or not Aβ peptides are capable of binding to the α_7 nAChR subtype. Using unlabelled $A\beta_{(1-42)}$, Wang *et al* (2000 a,b) have demonstrated the displacement of [^{125}I]α-BGT from α7SK-N-MC (full length human α_7 nAChR cDNA transfected in to the human neuroblastoma SK-N-MC cell line) membranes (Wang et al., 2000a) and [^{3}H]MLA from homogenized hippocampal or cortical tissue from rat brains (Wang et al., 2000b). These findings raise the notion that Aβ peptides may bind specifically to the α_7 nAChR subtype. However, the work presented in this chapter finds no evidence for such an interaction and therefore does not support the findings reported by Wang *et al* (2000 a, b). While [^{125}I]α-BGT was readily displaceable using α_7 nAChR selective ligands such as MLA and choline in both the homogenised and slice preparation, $A\beta_{(1-42)}$ or $A\beta_{(12-28)}$ were not capable of displacing [^{125}I]α-BGT binding under the same conditions. Interestingly, there was a considerable difference in the IC_{50} determined for choline in the homogenate (millimolar) and slice preparations (micromolar). Most likely

this is due to differences in the nature of the two preparations which affect choline binding. In slice preparations, but probably not membrane preparations, there remains a more integrated system with intact neuron-glia interactions, neuronal circuitry, metabolic processes and so forth.

The results presented here also indicate that *in vivo* chronic treatment of rats with $A\beta_{(1-40)}$ at a concentration which has been shown to impair cholinergic neuron function (Nitta et al., 1997; Nitta et al., 1994; Yamada et al., 1998) has no effect on the level of α_7 nAChRs or the expression of α_7 subunit mRNA. It has previously been demonstrated that chronic exposure to nicotinic agonists (Barrantes et al., 1995; Ke et al., 1998) and antagonists (Molinari et al., 1998) causes an up-regulation of the α_7 nAChR. In this context, the lack of change in α_7 nAChR levels following chronic $A\beta_{(1-40)}$ exposure shown here does not support the idea that Aβ peptides bind directly to these receptors. It is difficult to offer an obvious explanation for the difference in results between those reported here and those previously reported by Wang *et al* (2000 a, b). Possibly, any binding interaction between Aβ peptides and the α_7 nAChR is critically dependent on experimental conditions such as the aggregation state of the Aβ peptide. No attempt was made to assess the conformational state of the Aβ peptides prior to use. Despite this apparent inconsistency in results regarding the binding of Aβ peptides to α_7 nAChRs, there is certainly evidence to suggest that there is an interaction between the two proteins at some level. Dineley et al., (2001) have demonstrated that $A\beta_{(1-42)}$ in the picomolar–nanomolar range activates the mitogen-activated protein kinase cascade via α_7 nAChRs in the hippocampal slice preparation. However, in contrast to the results reported here which show that chronic *in vivo* treatment with $A\beta_{(1-40)}$ does not effect α_7 nAChR levels, Dineley et al., (2001) report that the α_7 nAChR receptor protein is up-regulated in cultured hippocampal slices following chronic (144 h) exposure to $A\beta_{(1-42)}$ *in vitro*. Collectively,

these results may highlight the importance of differences in the action of Aβ peptides *in vitro* and *in vivo* or perhaps differences in the activities of $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ in relation to α_7 nAChRs. Interestingly, Liu et al., (2001) have reported that $A\beta_{(1-42)}$ blocks the function of α_7 nAChRs non-competitively with an IC_{50} of 7.5 nM but does not displace [^{125}I]α-BGT binding up to a concentration of 200 nM under the same conditions. This finding is consistent with the results reported here in relation to the lack of displacement of [^{125}I]α-BGT by Aβ peptides. Furthermore, by recording single-channel currents from the soma of hippocampal Ca1 stratum radiutum interneurons, Pettit et al., (2001) have demonstrated that $A\beta_{(1-42)}$ reduces the probability and frequency of channel opening in both α_7 and non-α_7 nAChR receptor populations. Notably, this effect was significantly greater in the non-α_7 nAChR receptors and supports the finding that $A\beta_{(1-42)}$ may bind to multiple classes of nAChRs (Wang et al., 2000b). In fact, Aβ peptides have been shown to bind to a number of proteins including the low-density lipoprotein receptor-related protein (LRP) (for review see Hyman et al., 2000), apolipoproteinE (ApoE) (Pillot et al., 1999), scavenger receptors expressed in microglia and macrophages (El Khoury et al., 1996), the receptor for glycation end products (RAGE) (Yan et al., 1996) and endoplasmic reticulum amyloid binding protein (ERAB) (Yan et al., 1997). It is possible that due to the 'sticky' nature of Aβ peptides, they may bind non-specifically to a wide variety of proteins and this may be the basis for the interaction between $A\beta_{(1-42)}$ and the α_7 nAChR.

In conclusion, no evidence has been found for $A\beta_{(1-42)}$ or $A\beta_{(12-28)}$ binding to α_7 nAChRs up to a concentration of 30nM in coronal sections or hippocampal membrane fragments from rat brain. Consistent with this finding, chronic exposure to $A\beta_{(1-40)}$ *in vivo* does not affect α_7 nAChR receptor levels or subunit expression. The exact nature of the functional interaction between Aβ peptides and α_7 nAChRs previously reported (Dineley et al., 2001; Liu et al., 2001; Pettit et al., 2001) is an area that warrants further

investigation. The results presented here suggest that such an interaction is not likely to be mediated through the competitive binding of $A\beta_{(1-42)}$ to the α_7 nAChR.

Chapter 7

Transient knockdown of the α_7 nAChR subunit protein in the rat brain affects the expression of other nAChR subunits as well as certain proteins involved in the proteolytic processing of APP

7.1 INTRODUCTION

The α_7, α_8 and α_9 nAChR subunits are unique among nAChR subunits based on their capacity to form robust, homomeric nAChRs that are selectively sensitive to α-BGT when expressed in Xenopus oocytes. Although α_7 and α_8 subunits co-assemble in chicken nAChR (Gotti et al., 1994; Keyser et al., 1993), the apparent absence of the α_8 subunit in mammals, and the restricted localisation of the α_9 subunit (Park et al., 1997), has focussed attention on the α_7 subunit and corresponding homomeric receptor in mammalian neurons. The α_7 nAChR subtype is one of the most widely expressed nAChRs in the nervous system (for reviews see McGehee et al., 1995b; Sargent, 1993). These receptors have an unusually high relative permeability to calcium and regulate numerous calcium-dependant events in the nervous system (Bertrand et al., 1993; Seguela et al., 1993). Examples include transmitter release (Gray et al., 1996; McGehee et al., 1995a), second messenger cascades (Vijayaraghavan et al., 1995), neurite extension (Fu et al., 1998; Pugh et al., 1994), and both apoptosis (Berger et al., 1998) and neuronal survival (Messi et al., 1997). The α_7 nAChR can also contribute directly to postsynaptic currents (Chang et al., 1999; Ullian et al., 1997; Zhang et al., 1996) and are expressed at both somato-dendritic and presynaptic sites on neurons in the hippocampus (Alkondon et al., 1999; Frazier et al., 1998; Hefft et al., 1999), a structure critical for memory formation (Eichenbaum et al., 1999).

As described in detail throughout the work presented thus far, a consistent pathological feature associated with AD is the significant reduction in cerebral nAChR levels. While most studies have implicated the $\alpha_4\beta_2$ nAChR subtype with regard to this effect (Nordberg et al., 1986; Warpman et al., 1995; Whitehouse et al., 1986), there is some evidence, albeit inconsistent, to suggest that the α_7 nAChR subtype may also be involved. Specifically, post-mortem studies comparing the brains of AD subjects with age-

matched controls have reported either a loss (Burghaus et al., 2000; Guan et al., 2000; Wevers et al., 1999) or no significant change (Martin-Ruiz et al., 1999; Sugaya et al., 1990) in the level of α_7 nAChR subunit protein, while an increase in α_7 subunit expression at the mRNA level in AD brains has also been reported (Hellstrom-Lindahl et al., 1999). Clearly this is an area that warrants further investigation.

A more compelling case can be made for the significance and therapeutic relevance of the α_7 nAChR in AD based on studies which demonstrate neuroprotective and memory enhancing properties associated with this receptor subtype (Fujii et al., 2001; Levin et al., 1999; Matsuyama et al., 2000; Meyer et al., 1997; Rezvani et al., 2001). Activation of the α_7 nAChR has been reported to mediate neuroprotective effects against a variety of neurotoxic insults both in vitro and in vivo. Examples from in vitro studies include protection against trophic factor deprivation in PC12 cells (Li et al., 1999; Martin et al., 1994), as well as glutamate excitotoxicity (Kaneko et al., 1997) and Aβ induced neurotoxicity (Kihara et al., 1997 & 2000) in primary cortical and hippocampal neuronal cultures. Activation of α_7 nAChRs also promotes survival of spinal cord motorneurons from programmed cell death induced by trophic factor deprivation (Messi et al., 1997). Results from in vivo studies have demonstrated that the selective α_7 nAChR agonist, 3-(2,4-dimethoxybenzylidene)-anabaseine (DMXBA; code name GTS-21), protects against neocortical neuronal cell loss induced by nucleus basalis magnocellular lesions in rats (Meyer et al., 1998c; Nanri et al., 1998), and protects septal neurons from retrograde degeneration following unilateral fimbrial transections (Martin et al., 1994). Considering the selective degeneration of these neuronal populations seen in AD, these findings clearly have implications for the potential therapeutic benefit associated with α_7 nAChRs. It is not surprising then that clinical trials are underway for the treatment of AD using selective α_7 nAChR ligands such as GTS-21 (for review see Kem, 2000). Behavioural and electrophysiological studies that have investigated the involvement of α_7 nAChRs in

cognitive function and synaptic plasticity respectively, indicate that the therapeutic benefits of this receptor may extend beyond its neuroprotective properties. Activation of α_7 nAChRs has been found to enhance performance in several spatial and non-spatial memory-related paradigms (Meyer et al., 1997 & 1998b; Rezvani et al., 2001; Woodruff-Pak et al., 1994). Notably, Levin et al., (1999) has reported a significant reversal of the working memory impairments caused by basal forebrain lesions using the α_7 selective nAChR agonist AR-R 17779. Furthermore, a number of studies have demonstrated that activation α_7 nAChRs can promote LTP at glutamtergic synapses (Mansvelder et al., 2000; Matsuyama et al., 2000). As discussed in chapter 4, LTP is widely considered to represent the physiological basis of learning and memory.

A number of studies have reported that activation of the other major nAChR subtype in the CNS, the $\alpha_4\beta_2$ receptor, can also mediate both neuroprotection and positive effects in relation to learning and memory (for reviews see Belluardo et al., 2000; Rezvani et al., 2001). Thus, it seems likely that both the $\alpha_4\beta_2$ and α_7 nAChR subtypes may represent important therapeutic targets in for the treatment of AD. Of course, the significant loss of these receptor subtypes reported in AD brains may significantly limit their suitability as therapeutic targets. Based on the positive clinical effects of nicotine treatment on AD subjects despite this significant loss of nAChR density (Levin, 1992; Rezvani et al., 2001; Sahakian et al., 1989; Whitehouse et al., 1995), it is possible that additional nAChR subtypes may play important roles in learning and memory. It would certainly be useful to identify novel nAChR subtypes in this regard as targets for the development of selective ligands for the treatment of AD. One of the complicating factors associated with nAChR research is the lack of selective ligands available as tools for the study of the wide variety of nAChR subtypes likely to exist in the CNS. This makes it somewhat difficult to assess the therapeutic potential for these nAChR subtypes. One method which has been useful in determining the functional significance of specific

nAChR subunits in vivo has been the use of antisense oligodeoxyribonucleotide (ODN) probes which hybridise to the subunit mRNA thereby preventing translation, and transiently decreasing the expression of specific subunits (for reviews see Stein et al., 1993; Wagner, 1994). A study by Monia et al., (1993) reports that hybridisation of the ODN to the targeted mRNA may also facilitate the action of intracellular RNaseH and subsequent degradation of the ODN-mRNA hybrid. Regardless of the precise mechanism, the end result of inhibition of nAChR subunit mRNA translation will be reduced biosynthesis of the subunit protein and the corresponding receptor construct (fig. 7.0). This can also be achieved in transgenic 'knockout' mice in which the gene encoding the nAChR subunit in question has been deleted (knocked-out). Knockout animals differ from antisense ODN treated, or 'knockdown', animals in that the receptor subunit is lacking throughout development. It has been suggested that these animals display significant compensatory adaptations in neural circuitry in response to the loss of specific subunit proteins (Picciotto et al., 2000).

One of the aims of the work presented in this chapter has been to investigate whether there are homeostatic changes in the expression of other nAChR subunit mRNAs in response to transient knockdown of the α_7 nAChR subunit using an antisense ODN probe targeted to α_7 subunit mRNA (α_7ODN). Potentially, changes in other nAChR subunits may represent a homeostatic response in specific nAChR populations in an attempt to compensate for a loss of functions mediated by the α_7 nAChR subtype such as neuroprotection and cognition. By this reasoning, such nAChR subtypes may represent preliminary targets for nAChR based therapy of AD.

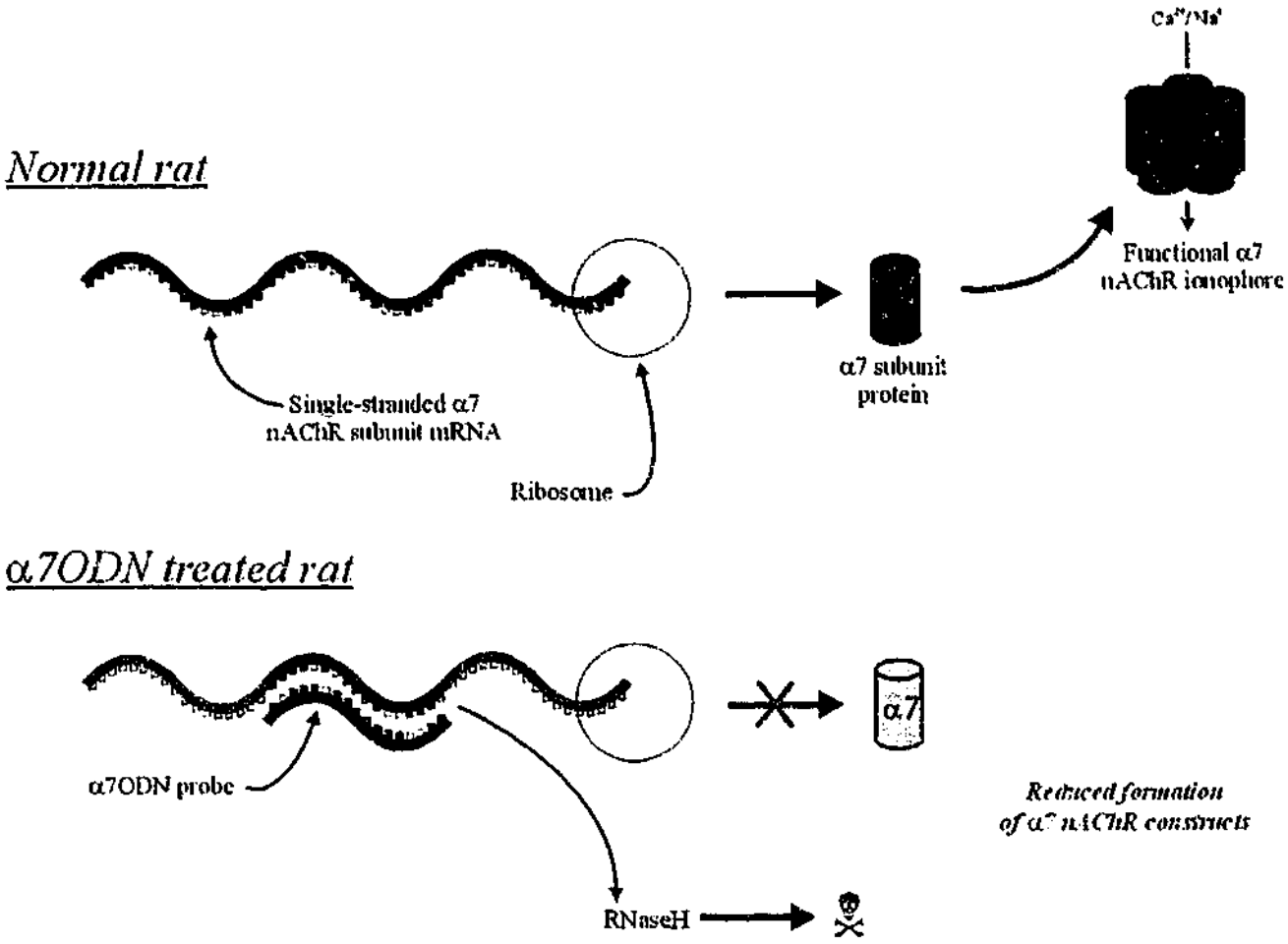


Figure 7.0. Schematic showing how a short antisense ODN probe targeted to specific mRNAs can be used to inhibit the synthesis of the corresponding protein – in this case the α_7 nAChR subunit protein. Normally, single stranded α_7 subunit mRNA synthesised in the cell nucleus is translated by ribosomes in the endoplasmic reticulum into the full α_7 subunit protein. Exposure to a short 20-mer ODN sequence complementary in base pair construction to a specific sequence in the α_7 mRNA results in hybridisation of the ODN to the α_7 mRNA. Consequently, the resulting double stranded section of the α_7 subunit mRNA cannot be translated by the ribosome, thereby preventing formation of the full α_7 subunit mRNA and the corresponding receptor constructs. Furthermore, degradation of the mRNA by intracellular RNaseH as a result of hybridisation may also contribute to decreased protein synthesis.

Another aim of the work presented here has been to use the same technique of transient α_7 subunit knockdown to investigate possible relationships between the α_7 nAChR population and the expression of APP695 and certain proteins required for its proteolytic processing including BACE, PS1, PS2 and TACE. A study by Kim et al., (1997) has reported that treatment of PC12 cells with nicotine increased the release of a carboxyl-terminally truncated, secreted form of APP (sAPP) into the conditioned medium without affecting the expression level of APP mRNA. While it has been widely reported that cholinergic transmission may be an important factor in APP processing through PKC coupled muscarinic receptor activation (for review see Rossner et al., 1998), this finding

indicates that nAChR activation may also play a role in APP metabolism. Little is known however, about which nAChR subtypes may be involved. Considering the widely reported changes in both nAChRs and Aβ levels in AD brains, establishing a relationship between nAChRs and APP processing will have important implications for the interpretation of the involvement of these two neurochemical markers with regard to the neurodegenerative process.

In summary, the aim of the work detailed in this chapter has been to investigate homeostatic responses in nAChR receptor populations and subunit levels as well as the expression of APP695, BACE, PS1, PS2 and TACE as a result of the transient inhibition (knockdown) of the α_7 nAChR subunit protein and consequently its corresponding homomeric receptor construct. The α_7 nAChR subunit was chosen based on well documented evidence describing the importance of the α_7 nAChR subtype in neuroprotection and cognitive function as well as therapeutic benefits in AD treatment. The relevance of the α_7 nAChR population to the expression of other nAChR subunits and the processing of APP has been discussed.

7.2 METHODS

7.2.1 TREATMENT OF ANIMALS AND TISSUE PREPARATION

The method relating to continuous i.c.v. infusion is based on that previously described in chapter 3, *3.2.1 Treatment of Animals and Tissue Preparation.*

Sprague-Dawley rats (250 – 300 g) of either sex were divided into 3 groups of 4 animals including an α_7 antisense ODN group, a mis-sense ODN group and a vehicle group. The mis-sense probe was used to control for non-specific effects associated with the i.c.v. infusion of an ODN but not related to the specific targeting of any known mRNA. Each rat was anaesthetised with pentobarbitone (60 mg/kg i.p.) and constrained to a tilted skull position (-0.3 mm) using a Kopf stereotaxic apparatus. All animals received continuous i.c.v. infusions for 7 days into the left, lateral ventricle (stereotaxic coordinates: 1.0 mm caudal to bregma, 1.5 mm left from the midline, 3.4 mm ventral from the cortical surface according to the atlas of Paxinos and Watson (1986). This was achieved using brain infusion kits (Alza corp., USA.) attached to modified miniosmotic pumps (pump rate of 1 µl/hr; Alza corp., USA). The infusion cannula was fixed to the skull using a stainless steel screw and dental cement and the osmotic minipump was placed between the scapulae in a small subcutaneous cavity. Treated animals received 0.167 µg/hr of α_7 nAChR subunit 20-mer antisense oligodeoxynucleotide (α_7ODN) dissolved in dH_2O. Controls received either vehicle (dH_2O) or 0.167 ug/hr of a mis-sense ODN (mis-ODN) that does not target any known mRNA in the rat brain as assessed through a BLAST search. The mis-ODN had the same Adenine:Cytosine:Thymine:Guanine ratio as the α_7ODN. Both the α_7ODN and the mis-ODN used phosphorothioated bases in order to prevent degradation of the ODNs

by intracellular nuclease activity. The continuous infusion of phosphorothioated ODNs via an osmotic mini-pump linked to an intra-ventricular cannula can maintain high concentrations of ODN in the CSF for several days and cause its relatively homogenous diffusion in the brain (Whitesell et al., 1993). The full sequence and accession details for the α_7ODN and mis-ODN are detailed below in table 7.0. Seven days following surgery the animals were decapitated and the brains frozen over liquid nitrogen. A Reichert Jung™ cryostat was used to collect a series of 14 μm sections from each brain. Consecutive coronal sections were taken at 3.6mm caudal to bregma, thaw mounted on poly-L-lysine coated slides and stored at -70°C for further use.

Target	Accession #	Sequence of ODN	Reference
α7KD	L31619	5'-GTGCTGGCGAAGTATTGTGC-3'	(Seguela et al., 1993)
MIS	n/a	5'-CACACACCTGACTGACTGAC-3'	

Table 7.0. Sequences and accession details for the α_7 and mis-sense ODNs used.

7.2.2 In Situ Hybridisation Histochemistry

The protocol for in situ hybridisation is based on the methods described Wisden et al., (1994) and adapted by Loiacono et al., (1999), and has been described in detail under General Methodology, *2.1 In Situ Hybridisation Histochemistry.*

7.2.3 [^{125}I]α-Bungarotoxin Autoradiography

The protocol for [^{125}I]α-BGT was based on that used by Whiteaker et al., (1999) and has been described in detail under General Methodology, *2.3 [^{125}I]α-Bungarotoxin Autoradiography.*

7.2.4 [^{3}H]EPIBATIDINE AUTORADIOGRAPHY

The protocol for [^{3}H]epibatidine autoradiography was based on that described by Perry et al., (1995b) and has been described in detail under General Methodology, *2.2 [^{3}H]Epibatidine Autoradiography*.

7.2.5 QUANTITATION

Images generated through the *in situ* hybridisation histochemistry and binding protocols were digitally captured and selected anatomical regions were quantified using Scion imaging software. For mRNA images, and [^{125}I]α-BGT autoradiographs the system was calibrated against a set of graded greyscales (Kodak), allowing for quantitation in terms of relative optical density (ROD). For [^{3}H]epibatidine autoradiographs, the system was calibrated against a set of standard tritium scales, allowing for quantitation in terms of nCi/mm^2. The mean ROD or nCi/mm^2 measurements for the vehicle treated groups was compared to the mean ROD or nCi/mm^2 for the mis-ODN and α_7ODN treatment groups using a one-way ANOVA with Dunnett's correction for multiple comparisons. Statistical significance was signified by P values of less then 0.5. The value in all treatment groups represents the mean of measurements taken from 4 animals. The symbol, '*', has been used to denote statistically significant differences between a particular treatment group and the vehicle treated group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Issues relating to quantitation have been described in greater detail in Chapter 2, General Methodology, *2.5 Quantitation*.

7.3 RESULTS

7.3.1 α_7 nAChR SUBUNIT mRNA EXPRESSION AND RECEPTOR LEVELS

The expression of α_7 nAChR subunit mRNA following continuous i.c.v. infusion of the α_7ODN was examined using *in situ* hybridisation histochemistry. The distribution and density of α_7 subunit mRNA in representative coronal sections from vehicle, α_7ODN and mis-ODN infused rats is shown in figure 7.1.

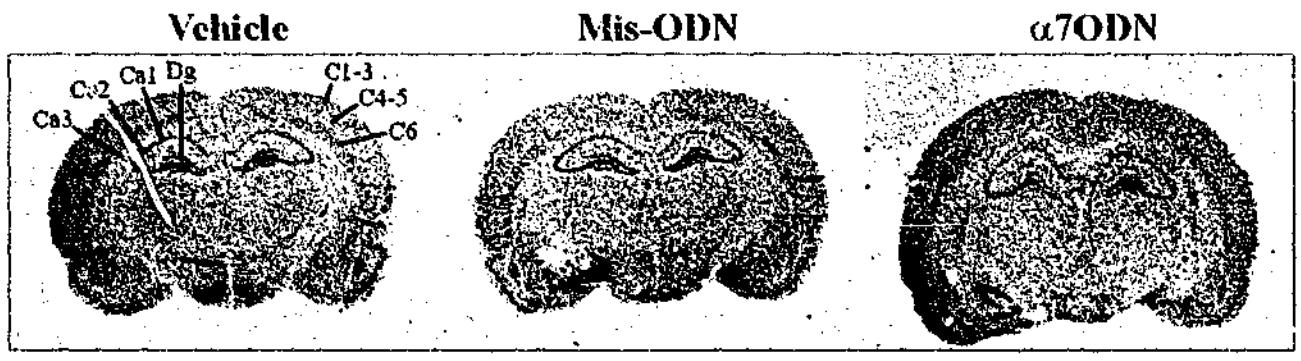


Figure 7.1. Distribution of the α_7 nAChR subunit mRNA in representative coronal sections approximately 3.6 mm caudal to bregma from rats that received a 7-day continuous i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C1-3, cortical layers 1 – 3; C4-5, cortical layers 4 – 5; C6, cortical layer 6; Dg, dentate gyrus; mis-ODN, mis-sense oligodeoxyribonucleotide. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Continuous i.c.v. infusion of the α_7ODN caused a significant reduction (44%) in the level of α_7 subunit mRNA expression in the dentate gyrus relative to the vehicle treated group (fig. 7.2). On the other hand, continuous infusion of the mis-ODN did not significantly affect α_7 mRNA expression relative to vehicle treated animals in any of the regions analysed.

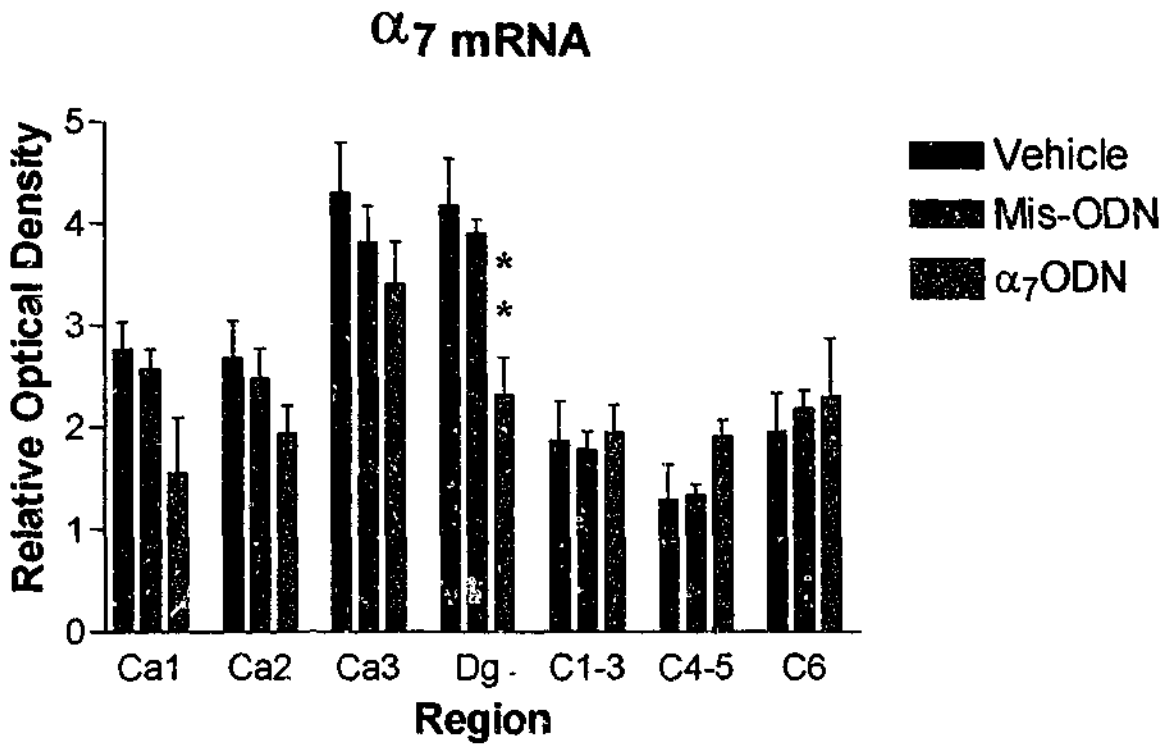


Figure 7.2. Levels of α_7 nAChR subunit mRNA expression, quantified in terms of relative optical density (ROD), in rats that received a continuous 7-day i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C1-3, cortical layers 1 – 3; C4-5, cortical layers 4 – 5; C6, cortical layer 6; Dg, dentate gyrus; mis-ODN, mis-sense oligodeoxyribonucleotide. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD for vehicle treated groups was compared to the mean ROD for the α_7ODN and mis-ODN treated groups using one-way ANOVA with a Dunnett post-test (**, $P < 0.01$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

The distribution and density of the mature α_7 nAChR in various cortical and hippocampal regions from rats receiving i.c.v. infusions of either vehicle, mis-ODN or α_7ODN was assessed by examining [^{125}I]α-BGT binding in coronal slices from each treatment group (fig. 7.3). Binding of [^{125}I]α-BGT to α_7 nAChRs was visibly reduced throughout the hippocampus and cortex in rats treated with a continuous i.c.v. infusion of the α_7ODN oligonucleotide. This effect was most pronounced in and around the immediate vicinity of the infusion site (left lateral ventricle).

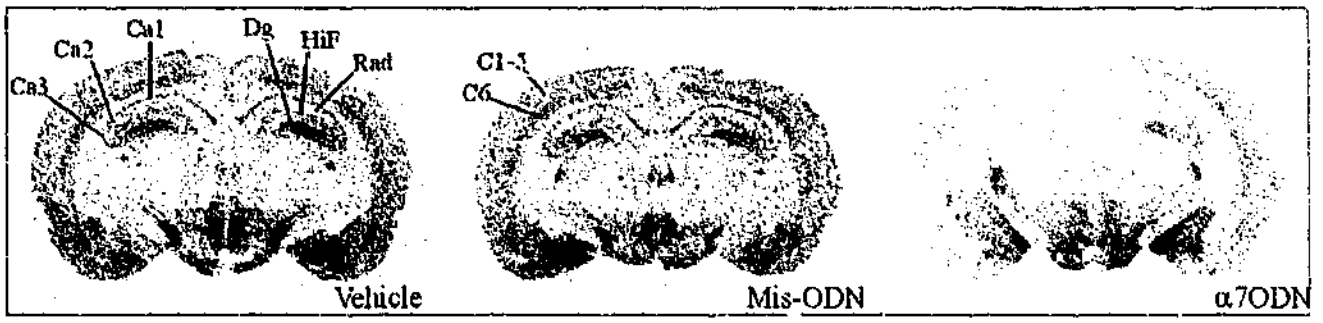


Figure 7.3. Total [^{125}I]α-BGT binding in representative coronal sections approximately 3.6 mm caudal to bregma from rats that received a 7-day continuous i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; mis-ODN, mis-sense oligodeoxyribonucleotide; Rad, stratum radiatum hippocampus.

The level of specific [^{125}I]α-BGT binding was significantly reduced by 54 – 68% throughout the hippocampus and cortex of α_7ODN treated rats relative to vehicle infused controls (fig 7.4). The mis-ODN, however, did not significantly affect the level of [^{125}I]α-BGT binding in these regions.

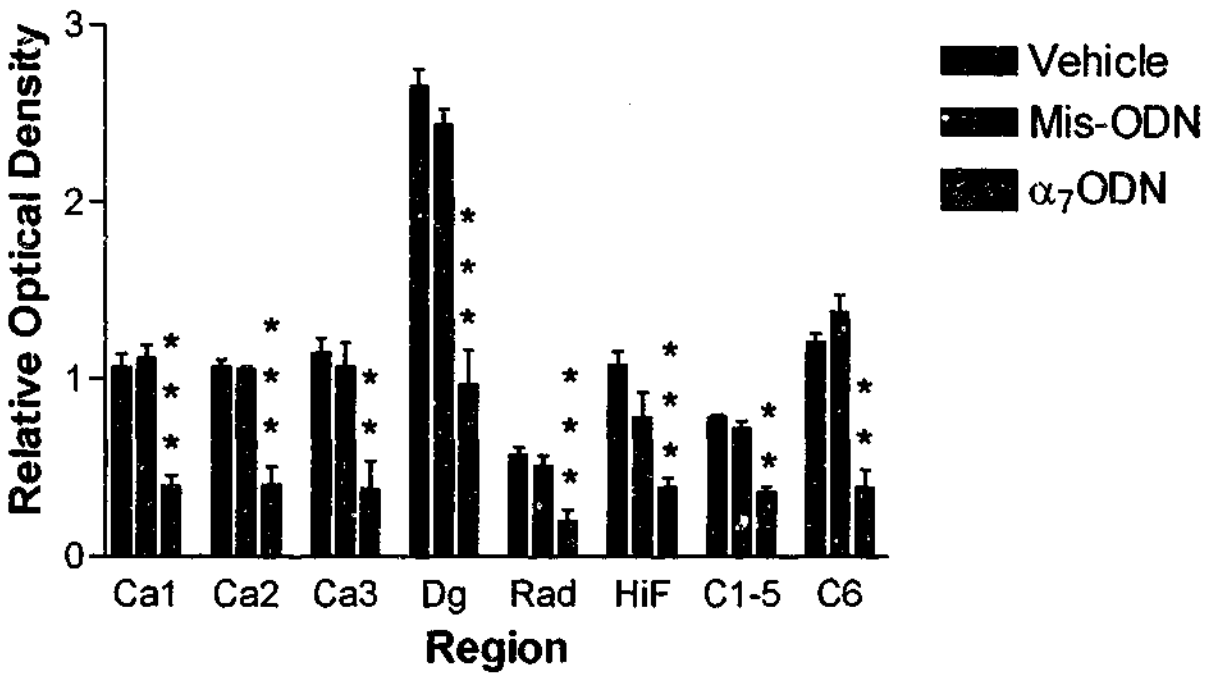


Figure 7.4. Specific [^{125}I]α-BGT binding, quantified in terms of relative optical density (ROD), in rats that received a 7-day continuous i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; mis-ODN, mis-sense oligodeoxyribonucleotide; Rad, stratum radiatum hippocampus. The mean ROD for vehicle treated groups was compared to the mean ROD for the α_7ODN and mis-ODN treated groups using one-way ANOVA with a Dunnett post-test (**, $P < 0.01$; *** $P < 0.001$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

7.3.2 NON-α_7 nACHR SUBUNIT mRNA EXPRESSION

The effect of inhibiting the translation of α_7 nAChR subunit mRNA into the α_7 subunit protein on the mRNA expression of other nAChR subunits was examined using *in situ* hybridisation histochemistry. The distribution and density of the α_3, α_4, α_5, α_6, β_2 and β_3 nAChR subunits in representative coronal sections from vehicle, α_7ODN and mis-ODN infused rats is shown in figure's 7.5 (alpha subunits) and 7.6 (beta subunits). The expression of α_4 and β_3 subunit mRNA was visibly increased in the dentate gyrus of rats treated with the α_7ODN.

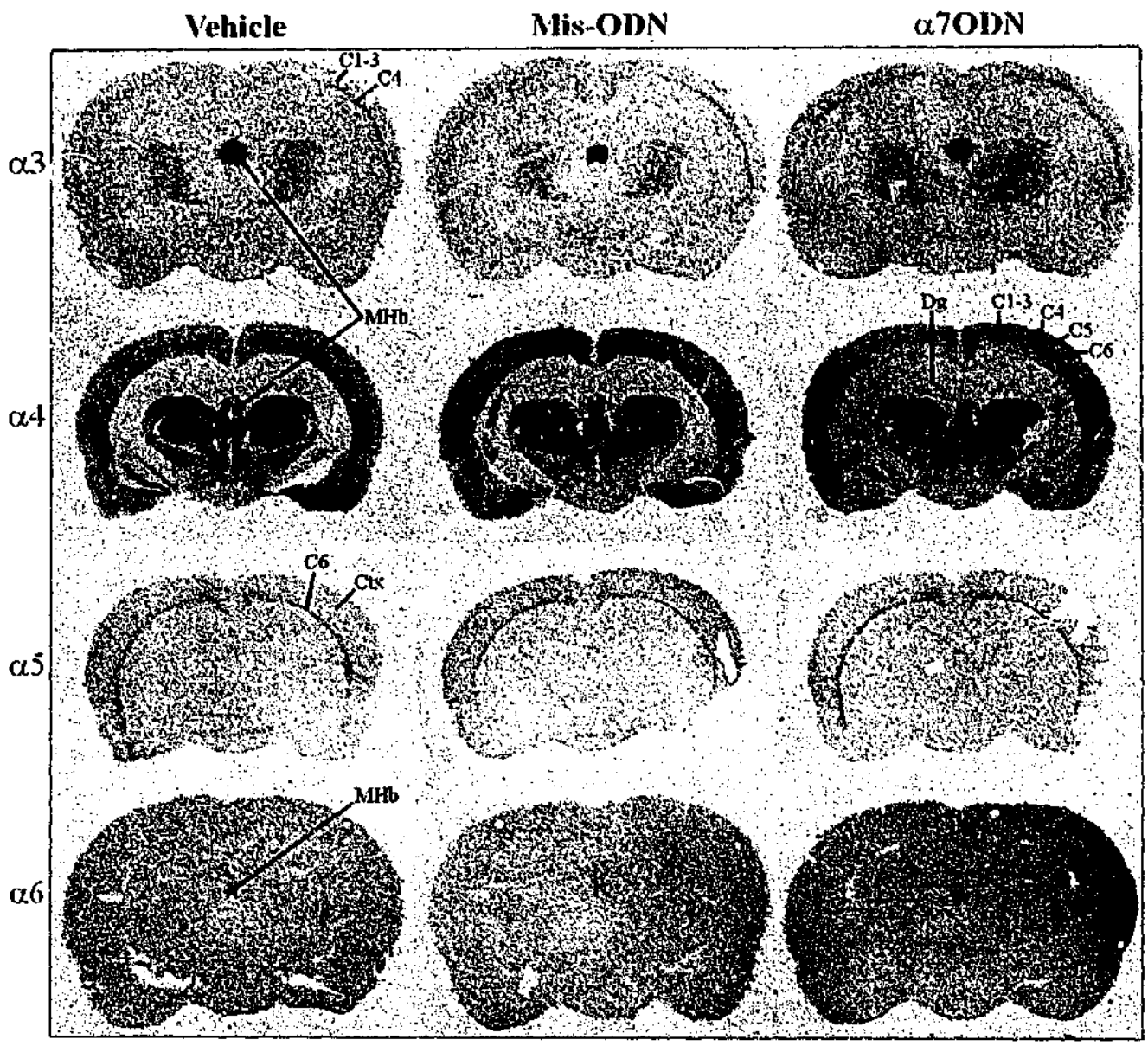


Figure 7.5. Distribution of the α_3, α_4, α_5 and α_6 nAChR subunit mRNAs in representative coronal sections approximately 3.6 mm caudal to bregma from rats that received a 7-day continuous i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C1-2, cortical layers 1 – 2; C1-3, cortical layers 1 – 3; C3, cortical layer 3; C4-5, cortical layers 4 – 5; C5-6, cortical layers 5 – 6; C6, cortical layer 6; Ctx, cortex; Dg, dentate gyrus; mis-ODN, mis-sense oligodeoxyribonucleotide; MHb, medial habenula. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

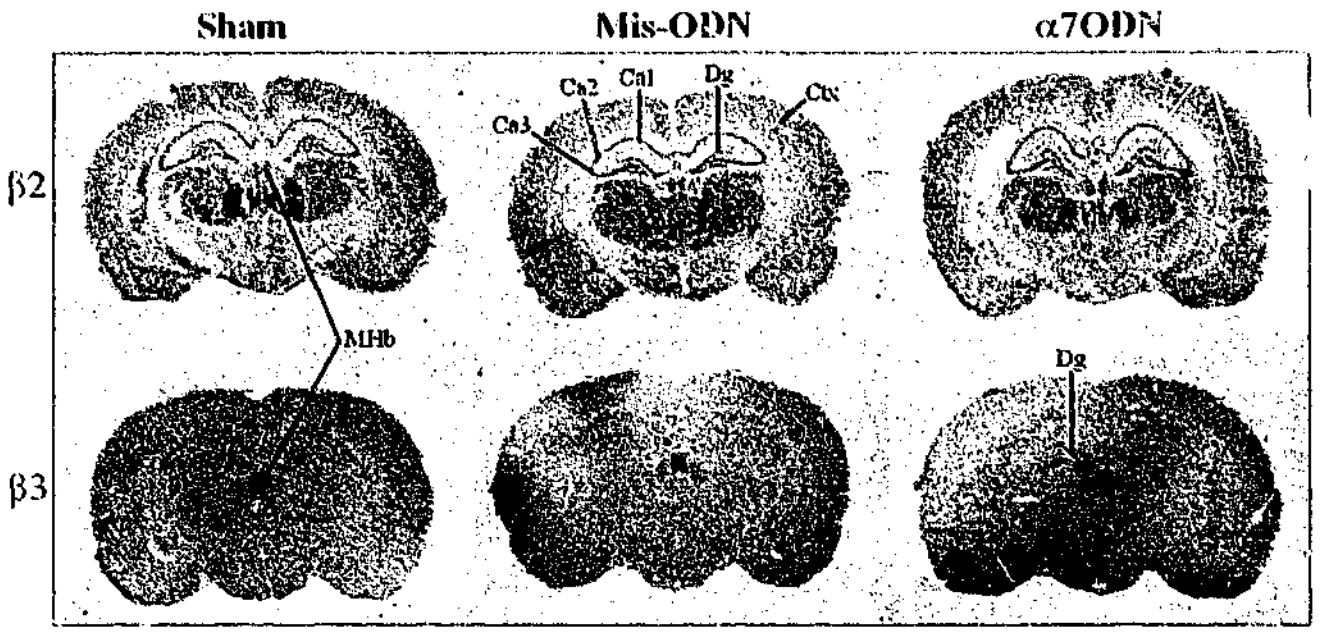


Figure 7.6. Distribution of the expression of the β_2 and β_3 nAChR subunit mRNAs in representative coronal sections approximately 3.6 mm caudal to bregma from rats that received a 7-day continuous i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; Ctx, cortex; Dg, dentate gyrus; mis-ODN, mis-sense oligodeoxyribonucleotide; MHb, medial habenula.

Relative to the vehicle treated group, in response to transient knockdown of the α_7 nAChR subunit the dentate gyrus showed a significant up-regulation in the expression of α_4 and β_3 nAChR subunit mRNA of 52 and 59% respectively (fig. 7.7). No change in the level of these receptor subunit mRNAs was seen in any of the other regions quantified. Rats treated with a continuous infusion of the mis-ODN probe did not show any significant change in the level of hippocampal or cortical expression of any of the nAChR subunit mRNAs examined in this study.

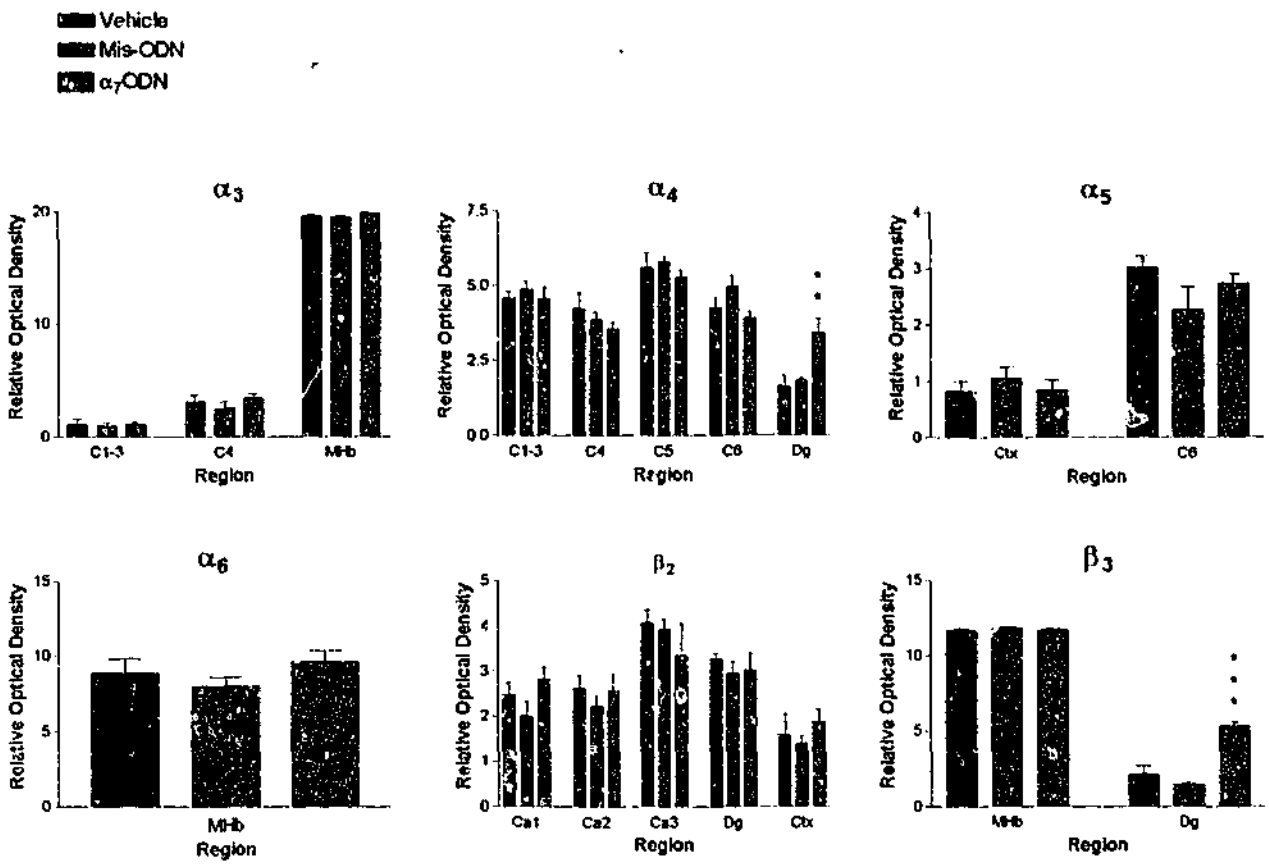


Figure 7.7. Levels of nAChR subunit mRNA expression, quantified in terms of relative optical density (ROD), in rats that received a 7-day continuous i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C1-2, cortical layers 1 – 2; C1-3, cortical layers 1 – 3; C3, cortical layer 3; C4-5, cortical layers 4 – 5; C5-6, cortical layers 5 – 6; C6, cortical layer 6; Dg, dentate gyrus; mis-ODN, mis-sense oligodeoxyribonucleotide; MHb, medial habenula. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD for vehicle treated groups was compared to the mean ROD for the α_7ODN and mis-ODN treated groups using one-way ANOVA with a Dunnett post-test (**, $P < 0.01$; ***, $P < 0.001$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

7.3.3 [^{3}H]Epibatidine Autoradiography

The distribution of [^{3}H]epibatidine binding, which reflects predominately the presence of the $\alpha_4\beta_2$ nAChR subtype, is shown in representative coronal sections from the vehicle, mis-ODN and α_7ODN treated groups in figure 7.8.

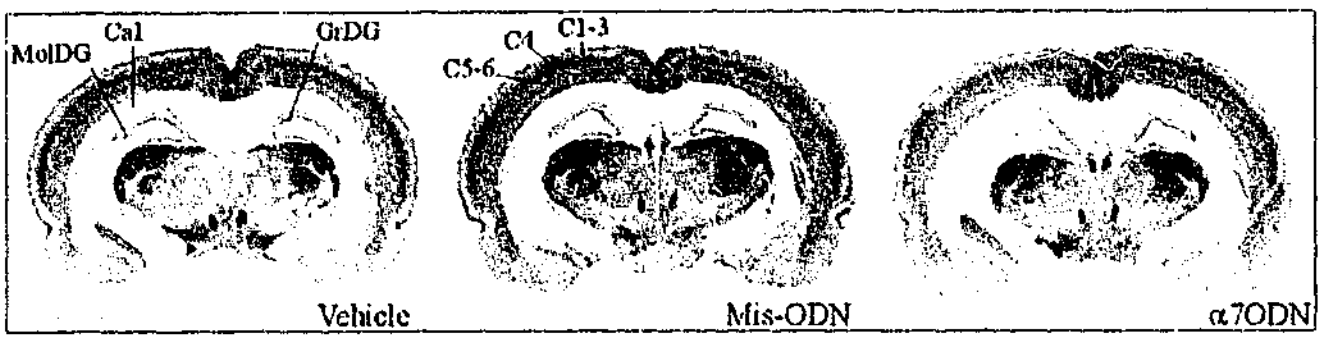


Figure 7.8. Total [^{3}H]epibatidine binding in representative coronal sections approximately 3.6 mm caudal to bregma from rats that received a 7-day continuous i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1, field of the hippocampus; C1-3, cortical layers 1 – 3; C4, cortical layer 4; C5-6, cortical layers 5 – 6; mis-OGN, mis-sense oligodeoxyribonucleotide; GrDG, dentate gyrus granular layer; MolDG, dentate gyrus molecular layer. Cortical layers defined as described in the atlas of Paxinos of Watson (Paxinos et al., 1986).

Densiometric assessment of the autoradiographic images from each treatment group revealed a significant reduction the cortical level of [^{3}H]epibatidine binding in rats treated with the α_7ODN relative to vehicle infused rats. Rats treated with the α_7ODN exhibited a significant reduction of 39%, 27% and 31% in cortical layers 1 -- 3, 4 and 5 – 6 respectively but showed no significant change in the hippocampal regions examined (fig 7.9). No significant changes were seen in the level of [^{3}H]epibatidine binding in rats treated with the mis-ODN in any of the regions examined.

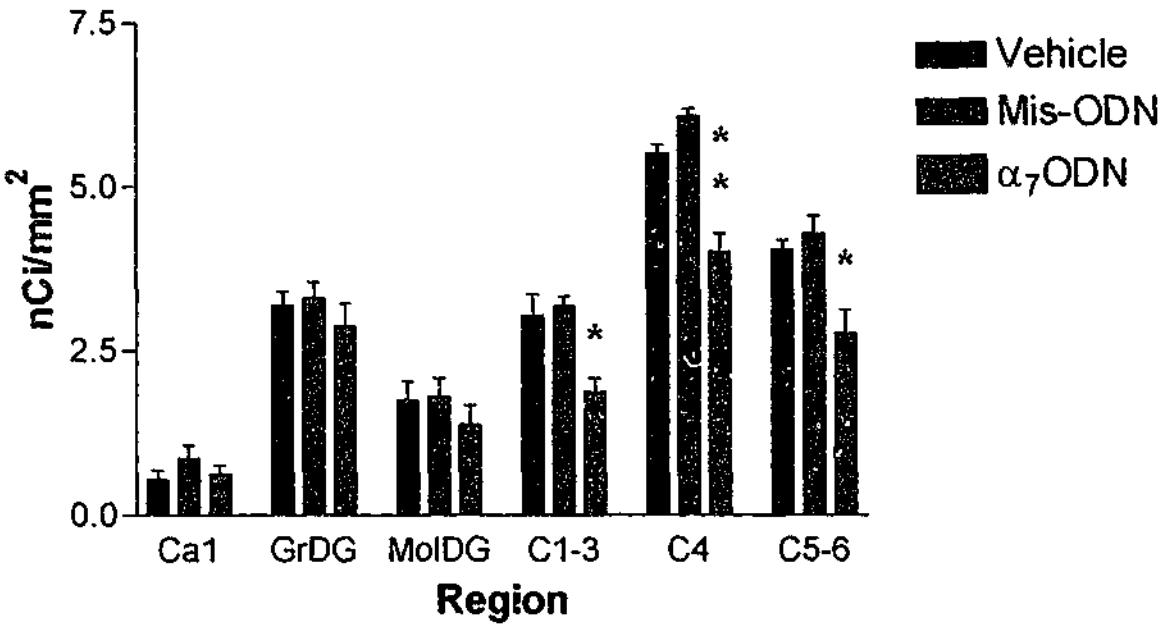


Figure 7.9. Specific [^{3}H]Epibatidine binding in selected cortical and hippocampal regions from rats that received a 7-day continuous i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1, field of the hippocampus; C1-3, cortical layers 1 – 3; C4, cortical layer 4; C5-6, cortical layers 5 – 6; GrDG, dentate gyrus granular layer; mis-ODN, mis-sense oligodeoxyribonucleotide; MolDG, dentate gyrus molecular layer. Cortical layers defined as described in the atlas of Paxinos of Watson (Paxinos et al., 1986). The mean nCi/mm^2 for vehicle treated groups was compared to the mean nCi/mm^2 for the α_7ODN and mis-ODN treated groups using one-way ANOVA with a Dunnett post-test (**, $P < 0.01$; **, $P < 0.01$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

7.3.4 mRNA EXPRESSION OF APP695, BACE, PS1, PS2 AND TACE

The mRNA expression of APP695 and a number of other proteins involved in the proteolytic processing of APP, including BACE, the presenilins and TACE, was examined in rats treated with i.c.v. infusions of either vehicle, mis-ODN or α_7ODN using *in situ* hybridisation histochemistry. The distribution of these mRNAs in representative coronal sections from vehicle, α_7ODN and mis-ODN treated rats is shown in figure 7.10.

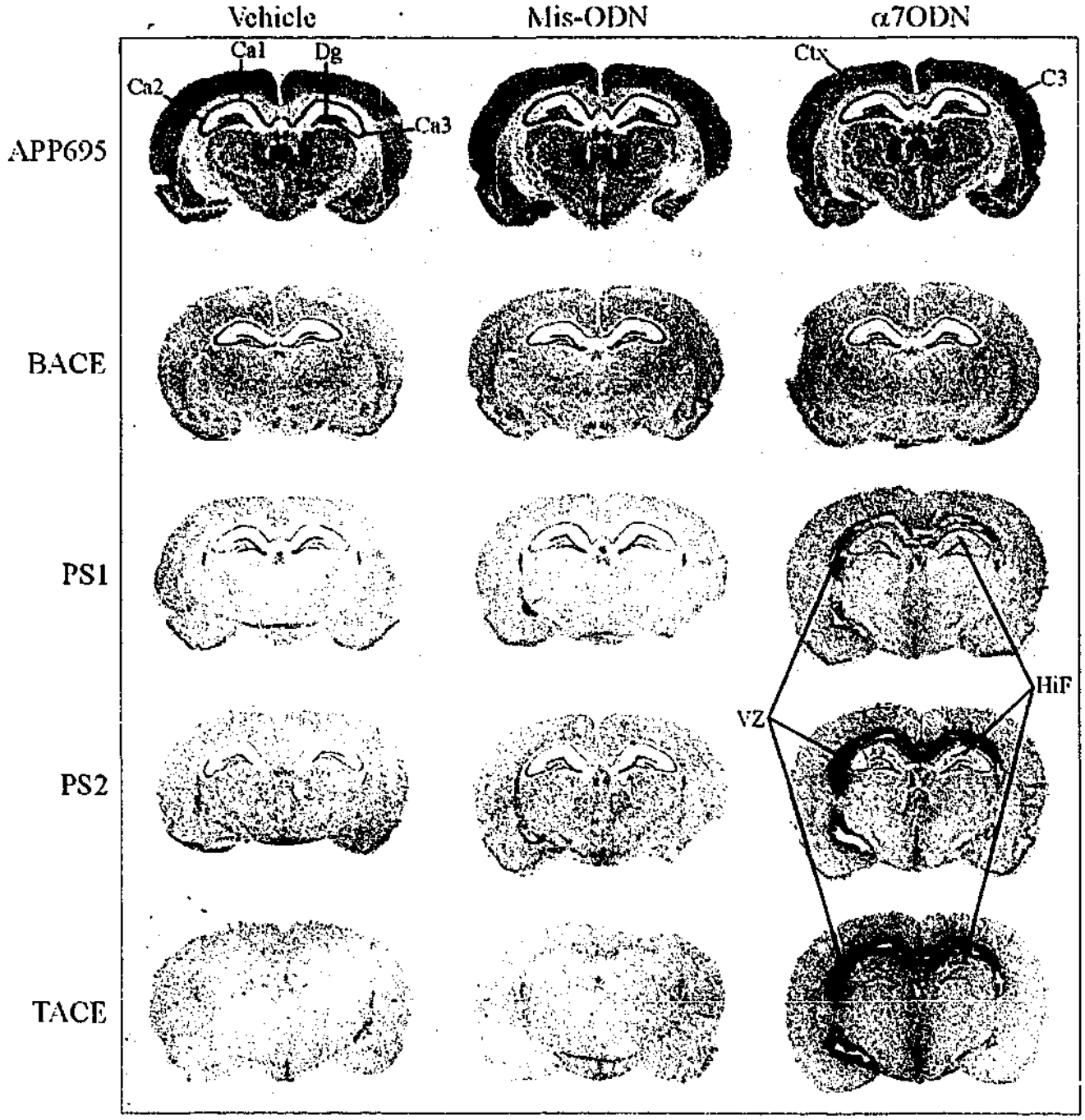


Figure 7.10. Distribution of APP695, β-site APP-cleaving enzyme (BACE), presenilin-1 (PS1), presenilin-2 (PS2) and tumour necrosis factor-α-converting enzyme (TACE) mRNA in representative coronal sections taken approximately 3.6 mm caudal to bregma from rats that received a 7-day continuous i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C3, cortical layer 3; Ctx, cortex; Dg, dentate gyrus; HiF, hippocampal fissure; mis-ODN, mis-sense oligodeoxyribonucleotide; VZ, ventricular zone.

Relative to the vehicle treated group, knockdown of the α_7 nAChR subunit caused a pronounced increase in the expression of PS1, PS2 and TACE mRNA in specific areas but did not affect the level of APP695 or BACE mRNA. There was a significant up-regulation in the expression of PS1, PS2 and TACE mRNA of 76 – 86% and 66 – 85% in the ventricular zone (VZ) cells and hippocampal fissure (HiF) respectively, in rats treated

with the α_7ODN (fig. 7.11). Additionally, the level of PS1 mRNA was also significantly increased by 33% in the cortex, while TACE mRNA levels were significantly increased by 52 – 83% throughout the hippocampus and cortex of the α_7ODN treated rats. Continuous i.c.v. infusion of the mis-ODN did not significantly affect APP695, BACE, PS1, PS2 or TACE levels in any of the regions examined relative to the vehicle treated group.

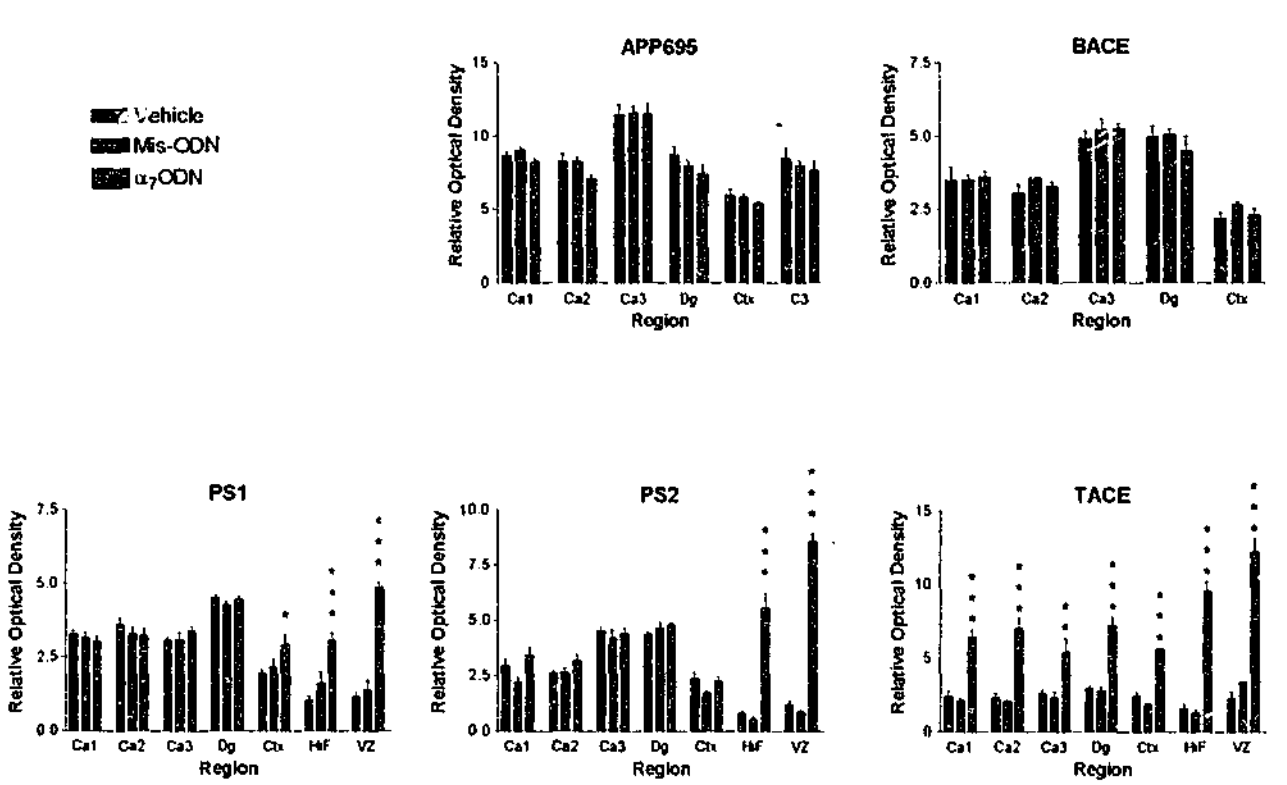


Figure 7.11. Levels of APP695, β-site APP-cleaving enzyme (BACE), presenilin-1 (PS1), presenilin-2 (PS2) and tumour necrosis factor-α-converting enzyme (TACE) mRNA, quantified in terms of relative optical density (ROD), in telencephic regions of rats that received a 7-day continuous i.c.v. infusion of either vehicle, α_7ODN or mis-ODN. Abbreviations: α_7ODN, α_7 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C3, cortical layer 3; Ctx, cortex; Dg, dentate gyrus; HiF, hippocampal fissure; mis-ODN, mis-sense oligodeoxyribonucleotide; VZ, ventricular zone. The mean ROD for vehicle treated groups was compared to the mean ROD for the α_7ODN and mis-ODN treated groups using one-way ANOVA with a Dunnett post-test (**, $P < 0.01$; **, $P < 0.01$; ***, $P < 0.001$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

7.4 DISCUSSION

The down-regulation in the biosynthesis of specific proteins by targeting the corresponding mRNAs with antisense ODNs is a novel approach for investigating the homeostatic changes that occur as a result of a loss of specific protein synthesis *in vivo*. It is not clear whether the predominate mechanism for reduced protein synthesis is due to inhibition of the translation of the hybridised section of mRNA by ribosomes in the endoplasmic reticulum, or the degradation of the ODN-mRNA hybrid resulting from the recruitment of intracellular RNaseH. There have been studies published which support both mechanisms of action. For instance, studies which show no change in the targeted mRNA level but a significant reduction in the corresponding protein following ODN treatment (Moats-Staats et al., 1993; Skutella et al., 1997) indicate that impaired ribosomal translation rather than RNaseH activity is likely to be important. A study by Monia et al., (1993), however, has reported that the efficacy of antisense ODNs in relation to inhibition of protein synthesis is highly correlated with the affinity of RNaseH for the ODN-mRNA hybridisation product. The results reported in this chapter, detailing the effects of *in vivo* treatment with an ODN probe targeted to the α_7 nAChR subunit mRNA, indicate that impaired translation and mRNA degradation are both likely to be important factors. Rats treated with the α_7ODN displayed a robust and nearly complete abolishment of [^{125}I]α-BGT binding throughout the hippocampus and cortex along with a more restricted decline in the α_7 mRNA signal which was limited to the granular layer of the dentate gyrus. Therefore, while the loss of [^{125}I]α-BGT binding in the dentate gyrus may be due to some degree of mRNA degradation by RNaseH, the loss of [^{125}I]α-BGT binding in areas in which there was a persistence of the α_7 mRNA signal probably reflects reduced α_7 protein synthesis primarily due to impaired ribosomal translation. Regional differences in the level

of α_7 mRNA reduction associated with i.c.v. antisense ODN probe infusion may be related to a number of reasons including variance in: the amount of probe which reaches different anatomical localities following infusion; the amount of residual mRNA levels in these areas following hybridisation of available antisense probe; and regional differences in the level of RNaseH activity.

One of the main aims of the work presented in this chapter has been to examine how inhibition of α_7 nAChR subunit protein synthesis might affect the mRNA expression of other nAChR subunits. It is reported here that rats treated with a continuous i.c.v. infusion of the antisense α_7ODN for 7 days exhibit a pronounced up-regulation of the expression of both α_4 and β_3 subunit mRNA in the granular layer of the dentate gyrus, in addition to a significant loss of α_7 mRNA expression and [^{125}I]α-BGT binding in this region. This response may reflect a homeostatic up-regulation of novel nAChR subtypes in an attempt to compensate for a loss of functions normally mediated by the α_7 nAChR. While the importance of the α_4 nAChR subunit in nAChR function has been well documented, relatively little is known about the functional significance of the β_3 subunit and some authors initially expressed doubts over it can form part of a functional nAChR (Deneris et al., 1989). Failure of β_3 subunit to form functional ion channels when expressed heterologously with other nAChR in *Xenopus* oocytes led to the labelling of β_3 as an 'orphan subunit' (Sargent, 1993). However, a number of studies have since reported evidence that suggests that β_3 subunits does in fact contribute to functional nAChR formation. For example, Groot-Kormelink et al., (1998) have reported that a mutated form of the human form of the β_3 subunit, which would be expected to increase agonist sensitivity, can form functional receptors when expressed in *Xenopus* oocytes along with the α_3 and β_4 subunits. This $\alpha_3\beta_3\alpha_4$ receptor has physiologically distinct properties when compare to the previously characterised $\alpha_3\beta_4$ subtype (Boorman et al., 2000). The finding

that β_3 subunit mRNA is selectively up-regulated in response to the inhibition of α_7 nAChR formation, reported here, supports the notion that the β_3 subunit may form part of functional nAChR subtypes. Interestingly, using intracellular recordings of voltage clamped *Xenopus* oocytes, Palma et al., (1999) have reported that the α_7 and β_3 nAChR subunits can co-assemble to form functional heteromemric receptors with physiological properties distinct from the homomeric α_7 receptor. Consistent with this finding, Matter et al., (1998) have characterised the promoters driving the transcription of the α_7 and β_3 nAChR subunit genes in chick retinal neurons and have reported that, although their regulatory modalities are different, they lead to co-expression of the α_7 and β_3 subunits in the same neurons. These findings, together with those reported here, indicate some degree of affiliation between the α_7 and β_3 nAChR subunits and raise the notion that these subunits may combine to form a novel nAChR subtype *in vivo*. Furthermore, the finding that there is a significant up-regulation of α_4 mRNA in rats treated with the α_7ODN suggests that a similar relationship may exist between the α_4 and α_7 receptor subunits. However, this subunit combination has not been shown to form functional nAChRs. Perhaps the most interesting hypothesis that might be advanced based on the results presented here, is that the selective increase in both β_3 and α_4 subunit mRNA in the dentate gyrus in response to a loss of α_7 subunit synthesis may represent a homeostatic up-regulation of a novel nAChR subtype incorporating both of these subunits – i.e. an $\alpha_4\beta_3$ nAChR subtype. Consistent with this idea, Vailati et al., (2000) have used subunit-specific antibodies in immunopurification experiments to show that the β_3 subunit is associated with the α_2, α_3, α_4, β_2 and β_4 subunits in a heterogeneous population of nAChRs which bind nAChR agonists with high (nM) affinity. Furthermore, Forsayeth et al., (1997) have demonstrated that the β_3 subunit is present in heteromeric receptors in rat cerebellar neurons together with the α_4, β_2 and β_4 subunits. The lack of change in [^{3}H]epibatidine

levels in the granular layer of the dentate gyrus following α_7ODN treatment suggests that the increase in α_4 mRNA expression in this region is not likely to reflect an up-regulation of the $\alpha_4\beta_2$ nAChR subtype. It is interesting to note that the up-regulation of α_4 and β_3 mRNA in α_7ODN treated rats was specifically restricted to the only region (the dentate gyrus) that also displayed a significant loss in α_7 mRNA levels. This suggests that the regulatory mechanism responsible for the homeostatic up-regulation of α_4 and β_3 mRNA is likely to be downstream of ribosomal translation and dependent on the cytoplasmic concentration of α_7 mRNA. On the other hand, in the cortex where there was no significant change in α_7 mRNA level but a significant decrease in α_7 receptor levels as a result of α_7ODN treatment, there was a significant reduction in [^{3}H]epibatidine binding. This result is somewhat more difficult to rationalise than the up-regulation of nAChR subunit mRNAs in the dentate gyrus and is an interesting response which may be directly related to the loss of α_7 nAChR density or, secondarily, to homeostatic changes in other nAChR populations following α_7 receptor loss. The lack of change in α_4 or β_2 subunit mRNA levels in the cortex suggests that the loss in [^{3}H]epibatidine binding in this region, following α_7ODN treatment, is likely to be mediated at a post-transcriptional level. There have been a number of studies which report that both the $\alpha_4\beta_2$ (Nordberg et al., 1986; Warpman et al., 1995; Whitehouse et al., 1986) and α_7 (Burghaus et al., 2000; Guan et al., 2000; Wevers et al., 1999) nAChR subtypes are lost as part of the neurodegenerative process in AD. The findings presented here with regard to the loss of [^{3}H]epibatidine binding in response to an inhibition of α_7 subunit synthesis raise the interesting notion that the loss of $\alpha_4\beta_2$ nAChRs in AD may be related to and even caused by an initial loss in α_7 nAChR density. Therefore, the loss of α_7 nAChR density in AD may be an important pathological change with implications beyond those associated specifically with a loss of α_7 nAChR function. As far as can be determined there has been no data previously reported describing the

regulation of other nAChR populations by α_7 nAChR numbers. A useful direction in future work may involve investigating the effect of transiently inhibiting α_4 or β_3 mRNA on the α_7 nAChR population.

In summary, with regard to nAChRs, transient inhibition of the synthesis of the α_7 nAChR subunit protein following i.c.v. infusion of an ODN targeted to α_7 mRNA results in a significant loss of α_7 nAChR density and a concomitant increase in α_4 and β_3 nAChR mRNA. Infusion of the mis-ODN did not significantly affect nAChR mRNA or receptor levels and therefore the changes associated with α_7ODN treatment are likely to be related specifically to impaired translation of α_7 mRNA rather then non-specific affects associated with i.c.v. ODN infusion. The selective and localised increase in α_4 and β_3 mRNA may represent a homeostatic up-regulation of novel nAChR subtypes with similar functional properties to the α_7 nAChR. Considering the potential benefit of α_7 nAChR activation in the treatment of AD along with reports that there may be a significant loss of these receptors in AD brains, targeting nAChR populations which persist during AD neurodegeneration and have functional properties similar to the α_7 subtype represents a reasonable approach to the development of new nicotinic based therapies. In this context, the work presented here details preliminary evidence that novel nAChR subtypes incorporating the α_4 and β_3 subunits represent potential therapeutic targets.

In addition to changes in nAChR markers, i.c.v. infusion of the α_7ODN caused significant changes in the mRNA expression of PS1, PS2 and TACE mRNA in specific anatomical regions. As has been discussed, these proteins play an important role in the secretion of sAPP fragments following the proteolytic processing of full length APP. Two proteolytic pathways have been characterised for APP metabolism that differ in terms of the sAPP fragments produced due to variability in the cleavage sites (refer to fig. 1.1, Chapter 1, *General Introduction*). The amyloidogenic β-secretase pathway involves an

initial cleavage by BACE at the β-secretase site at the N-terminal end of the Aβ sequence followed by PS1/2 dependent cleavage at the γ-secretase site at the C-terminal end of the Aβ sequence. The result is the formation of Aβ as well as a C-terminally truncated soluble APP fragment, sAPPβ. Alternatively, APP may be processed along the non-amyloidogenic α-secretase pathway which involves cleavage within the Aβ sequence at the α-secretase site in a process which is mediated to some degree by TACE, followed by PS1/2 mediated cleavage at the γ-secretase site. The resulting soluble APP fragments include a small section of the Aβ sequence of unknown function termed 'p3' as well as the longer, C-terminally truncated sAPPα fragment. Following α_7ODN treatment, the significant increase in PS1, PS2 and TACE but not BACE mRNA reported here, reflects the specific up-regulation of mRNAs for the proteins that are involved in the non-amyloidogenic α-secretase mediated processing of APP and subsequent generation of sAPPα. A study by Kim et al., (1997) has reported that nAChR stimulation can stimulate the processing of APP and the release of sAPPα in a dose dependent manner. Considering the affinity of nicotine in rat brain varies from nM for the $\alpha_4\beta_2$ nAChR to μM for the α_7 nAChR subtype, the required (–)-nicotine dose of >50 μM is consistent with the activation of the α_7 rather then $\alpha_4\beta_2$ subtype as being necessary for the processing of APP. The mechanism of action is not clear at present but is likely to be dependent on $[Ca^{2+}]_i$ considering that the effect of nicotine on APP processing is almost completely abolished by the calcium chelator, EGTA. This is consistent with a number of studies which have demonstrated that APP processing is stimulated by increases in $[Ca^{2+}]_i$ (Buxbaum et al., 1994; Loffler et al., 1993; Nitsch et al., 1993; Querfurth et al., 1994). Nicotinic receptors, and particularly the α_7 receptor subtype, are well suited to mediating cellular events dependent on $[Ca^{2+}]_i$ levels based on the high permeability of these receptors to Ca^{2+} ions. Certain neurotrophic actions facilitated by α_7 nAChR activation, including survival of spinal cord motorneurons

following trophic factor deprivation, are mediated by Ca^{2+} influx (Messi et al., 1997). Although relatively little is known about the biological function of APP, sAPPα has been shown to possess potent trophic and neuroprotective activity in a variety of cell culture models (Goodman et al., 1994a; Mattson et al., 1993a; Schubert et al., 1989b). Kim et al., (1997) and colleagues have raised the interesting hypothesis that the neuroprotective effects associated with nAChR stimulation may be mediated by the release of secreted APP fragments such as sAPPα. One possible interpretation of the results presented here with regard to the up-regulation of mRNA expression of proteins specifically involved in sAPPα production following α_7ODN treatment, is that this effect represents a homeostatic response to a loss of α_7 nAChR mediated sAPPα secretion. Up-regulation of TACE, PS1 and PS2 levels may reflect the mechanism by which a certain level of sAPPα secretion is maintained in the brain. Consistent with this idea, Kim et al., (1997) report that the increased sAPPα production associated with nAChR stimulation is not associated with an increase in APP expression and is more likely to reflect an increase in the processing of existing APP by the secretase enzymes. The finding that TACE mediated cleavage of APP is a calcium activated, reported by Allsop et al., (1991), supports the notion that nAChR stimulation may facilitate APP processing through increased activity of the proteins involved in its proteolytic cleavage.

Interestingly, the increase in TACE, PS1 and PS2 in α_7ODN treated rats was most pronounced in the cells of the VZ. If one considers the aforementioned neurotrophic activities associated with α_7 nAChRs and secreted forms of APP, this result is perhaps consistent with evidence demonstrating the importance of VZ cells in neurogenesis (for reviews see Bruni, 1998; Goldman, 1998; Kuhn et al., 1999; Morshead et al., 2001). Considerable progress has been made over the last decade regarding the fundamental properties of neural stem cells in the mammalian adult brain. Recently, there has been much interest in the identification and precise location of the neural stem cells *in vivo* and

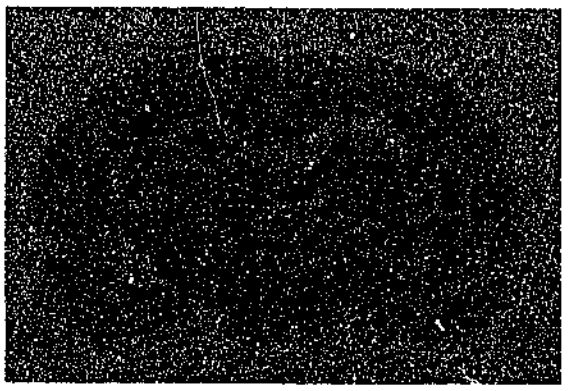
it has been demonstrated that there are two distinct locations within the adult brain: the ependymal layer lining the ventricles, and the subependymal layer immediately adjacent to the ependyma (Morshead et al., 2001). The generation and survival of new neurons from these cells is regulated by trophic factors such as brain derived neurotrophic factor (BDNF) and fibroblast growth factor-2 (FGF-2) (Benraiss et al., 2001; Pincus et al., 1998). Furthermore, it has been demonstrated that secreted APP enhances the proliferation of neural stem cells and that the N-terminal region of APP is likely to be responsible for this action (Hayashi et al., 1994; Ohsawa et al., 1999). Together with the report that ependymal cells are positive for antibodies directed against the N-terminal region of APP (Coria et al., 1992), these findings suggest that secreted forms of APP, such as sAPPα, play in important role in the development and maintenance of neuronal precursor cells in the VZ. One explanation for the significant increase in PS1, PS2 and TACE mRNA in the VZ of α_7ODN treated rats reported here is that it may reflect a homeostatic attempt to maintain a certain level of sAPPα secretion in this region in response to a reduced level of α_7 nAChR mediated processing of APP. In support of this theory, a study by Kostyszyn et al., (2001) has reported PS1 immunoreactivity in neuroblasts localised to the neuroepithilial cell layer of the VZ and has suggested that presenilin-1 may also be involved in processes such as axonal and dendritic outgrowth or synaptic formation. Furthermore, Rogers et al., (2001) have demonstrated that oligodendrocyte precursor cells (OPCs) purified from the rat corpus collusum express a number of nAChR subunits including the α_7 nAChR subunit, and approximately 65% of OPCs loaded with the calcium-responsive dye FURA-2 increased their intracellular free calcium in response to nicotine application.

The work presented in this chapter regarding the increase in PS1/2 and TACE in α_7ODN treated rats provides important evidence suggesting that activation of the α_7 nAChR subtype may be an important factor in the regulation of APP processing. Furthermore, based on the localisation of changes in PS1, PS2 and TACE mRNA, this

evidence circumstantially supports the theory that the neurotrophic activity associated with α_7 nAChR activation may be related to the secretion of sAPPα. Accordingly, a loss of α_7 nAChR density associated with AD may be a direct cause of further neurodegenerative changes or may increase the vulnerability of certain neuronal populations to neurotoxic insults such as Aβ exposure. Notably, neuronal damage associated with Aβ exposure can be significantly attenuated by both nAChR stimulation (for reviews see Shimohama et al., 2001; Zamani et al., 2001) and sAPPα (Goodman et al., 1994a). A useful direction for future work in this area may involve investigating the effects of transient knockdown of the α_7 nAChR on markers for neurogenesis and measures of sAPPα dependent neuroprotection. It would also be interesting to examine the actions of α_7 selective agonists currently in clinical trials for the treatment of AD in this respect.

Chapter 8

Transient knockdown of the APP695 protein in the rat brain affects α_7 nAChR-density as well as the expression of TACE mRNA in certain telencephic areas



8.1 INTRODUCTION

A central theme in the amyloid cascade theory of AD pathology states that one of the causative factors associated with AD neurodegeneration is the accumulation of Aβ as a result of an imbalance between its production and clearance in the CNS. Consequently, a considerable amount of work has focussed on rationalising a pharmacological intervention on the processing of APP in an attempt to limit Aβ production as a therapeutic strategy in AD. As a result, inhibitors of the γ and β-secretase enzymes, which together process APP along the amyloidogenic pathway are currently being developed for use in clinical trials by a number of pharmaceutical companies including Amgen, Bristol-Meyer Squibbs, Elan Pharmaceuticals, Scios Inc. and SmithKline Beecham. However, despite extensive chemical and biological scrutiny, relatively little is known about the precise physiological role of APP or its metabolic products.

The first function to be described for APP involved the identification of the Kunitz protease inhibitor (KPI) motif in the sequence of the APP751 and APP770 isoforms as an inhibitor of certain serine proteases such as trypsin and chymotrypsin (Kitaguchi et al., 1988; Oltersdorf et al., 1989; Sinha et al., 1990; Van Nostrand et al., 1989). Another possible function for the secreted ectodomain of APP is as an extracellular substrate promoting cell-cell or cell-matrix interactions (Schubert et al., 1989a). Several studies have demonstrated that secreted forms of APP may mediate cell-cell or cell-substrate adhesion in cultures of primary neurons or neuronal like cell lines (Breen et al., 1991; Chen et al., 1991; Milward et al., 1992; Schubert et al., 1989a). As discussed in the previous chapter, secreted full length APP and its metabolic products, particularly sAPPα, have been shown to possess potent neurotrophic and neuroprotective activities. Primary rat hippocampal neurons grown on a monolayer of APP-transfected Chinese hamster ovary CHO cells show enhanced neurite outgrowth compared with neurons grown on non-transfected CHO cells

(Qiu et al., 1995). Furthermore, Saitoh et al., (1989) have reported that fibroblasts treated with antisense ODN targeted towards APP mRNA show a marked decrease in APP expression and concomitant decrease in proliferation. Cell growth could be restored by the addition of exogenous, purified sAPP. In addition to stimulating neuronal growth and survival there have been a number studies published that describe the neuroprotective effects of secreted forms of APP in a variety of neurodegenerative paradigms. For example, *in vitro*, sAPPs can protect cultured neurons from neuronal damage caused by excitotoxicity and hypoglycaemia (Mattson et al., 1993a), ischemia (Smith-Swintosky et al., 1994), or Aβ mediated neurotoxicity (Goodman et al., 1994a). *In vivo*, moderate over-expression of sAPPs in transgenic mice confers resistance to acute and chronic forms of excitotoxicity, and has a synaptotrophic effect (Masliah et al., 1997; Mucke et al., 1994 & 1996). While the precise mechanism of action is not clear, the neurotrophic and neuroprotective properties associated with sAPPs is thought to be related to stabilisation of the intraneuronal concentration of calcium (Li et al., 2000; Mattson et al., 1993a & 1993b). A study by Furukawa et al., (1996a) has reported that sAPPs may be involved in the containment of neuronal excitability based on the finding that activation of a high conductance charybdotoxin-sensitive K^+-channel by sAPPs leads to hyperpolarisation of neurons and reduced intracellular calcium concentrations. Of course, the corollary of the finding that sAPPs maintain neuroprotection by stabilising $[Ca^{2+}]_i$, is that a loss of sAPP levels may be expected to predispose neurons to Ca^{2+} sensitive forms of neuronal damage such as excitotoxicty and Aβ toxicity. In support of this hypothesis, decreased levels of soluble APPs have been found in the cerebrospinal fluid of AD patients (Van Nostrand et al., 1992), and accumulating evidence suggests that altered regulation of $[Ca^{2+}]_i$ may play a role in the neuronal damage that occurs in AD (Gibson et al., 1987; Greenamyre et al., 1989; Mattson et al., 1992; Siesjo et al., 1989).

Consistent with the notion that a loss of sAPPs may predispose neurons to damage caused by excessive neuronal excitability and $[Ca^{2+}]_i$ overload, a study by Steinbach et al., (1998) has shown that transgenic mice either homozygous for a targeted mutation of the APP gene, or a deletion of the entire APP locus, display a significantly increased level of cortical neuronal excitability as well as an increased sensitivity to kainic acid induced seizures. Relative to wild-types, these mice displayed seizure activity which started earlier, was more severe, and led to enhanced mortality. During the initial stages of sporadic AD, seizures are usually considered relatively infrequent, but tend to become more frequent toward the end of the clinical course (Hauser et al., 1986; Romanelli et al., 1990). Interestingly though, familial AD caused by APP or PS1 mutations is often associated with seizures and myclonic jerks early in the progression of the disease (Haltia et al., 1994), affecting more than 80% of individuals in some pedigrees (Lampe et al., 1994). This corresponds with pronounced mis-processing of APP and, specifically, a reduction of processing along the non-amyloidogenic pathway and subsequent secretion of the neuroprotective sAPPα fragment (Lannfelt et al., 1995).

Based on the aforementioned findings that indicate a loss of sAPP levels may contribute to neuronal damage in AD, the aim of the work presented in this chapter has been to investigate the effect of inhibiting the synthesis of APP on a number of other neurochemical markers relevant to AD pathology. Specifically, nAChR density and the expression of various nAChR subunit mRNAs as well as mRNA for APP695, BACE, PS1, PS2 and TACE were examined following i.c.v. infusion of rats with an antisense ODN targeted to APP.

8.2 METHODS

8.2.1 TREATMENT OF ANIMALS AND TISSUE PREPARATION

The method relating to continuous i.c.v. infusion is based on that previously described in the previous chapter 3, *3.2.1 Treatment of Animals and Tissue Preparation.*

Sprague-Dawley rats (250 – 300 g) of either sex were divided into 3 groups of 4 animals including an APP695 antisense ODN group, a mis-sense ODN group and a vehicle group. Each rat was anaesthetised with pentobarbitone (60 mg/kg i.p.) and constrained to a tilted skull position (-0.3 mm) using a Kopf stereotaxic apparatus. All animals received continuous i.c.v. infusions for 7 days into the left, lateral ventricle (stereotaxic coordinates: 1.0 mm caudal to bregma, 1.5 mm left from the midline, 3.4 mm ventral from the cortical surface according to the atlas of Paxinos and Watson (1986). This was achieved using brain infusion kits (Alza corp., USA.) attached to modified miniosmotic pumps (pump rate of 1 µl/hr; Alza corp., USA). The infusion cannula was fixed to the skull using a stainless steel screw and dental cement and the osmotic minipump was placed between the scapulae in a small subcutaneous cavity. Treated animals received 0.167 µg/hr of a 20-mer antisense oligodeoxynucleotide targeted to APP695 mRNA (APP-ODN) dissolved in dH_2O. Controls received either dH_2O alone or 0.167 ug/hr of a mis-sense ODN (mis-ODN) that does not target any known mRNA in the rat brain as assessed through a BLAST search. The mis-ODN had the same Adenine:Cytosine:Thymine:Guanine ratio as the APP-ODN. Both the APP-ODN and the mis-ODN used phosphorothioated bases in order to prevent degradation of the ODNs by intracellular nuclease activity. The continuous infusion of phosphorothioated ODNs via an osmotic mini-pump linked to an intra-ventricular cannula

can maintain high concentrations of ODN in the CSF for several days and cause its relatively homogenous diffusion in the brain (Whitesell et al., 1993). The full sequence and accession details for the APP-ODN and mis-ODN are detailed below in table 8.0. Seven days following surgery the animals were decapitated and the brains frozen over liquid nitrogen. A Reichert Jung™ cryostat was used to collect a series of 14 µm sections from each brain. Consecutive coronal sections were taken at 3.6mm caudal to bregma, thaw mounted on poly-L-lysine coated slides and stored at -70°C for further use.

Target	Accession #	Sequence of ODN	Reference
APPKD	AY011335	5'-GGTGCCAATGCAGGTTTTGG-3'	(Murphy et al., 2001)
MIS	n/a	5'-CACACACCTGACTGACTGAC-3'	

Table 8.0. Sequences and accession details for the APP and mis-sense ODNs used.

8.2.2 In Situ Hybridisation Histochemistry

The protocol for in situ hybridisation is based on the methods described Wisden et al., (1994) and adapted by Loiacono et al., (1999), and has been described in detail under General Methodology, *2.1 In Situ Hybridisation Histochemistry*.

8.2.3 [^{3}H]Epibatidine Autoradiography

The protocol for [^{3}H]epibatidine autoradiography was based on that described by Perry et al., (1995b) and has been described in detail under General Methodology, *2.2 [^{3}H]Epibatidine Autoradiography*.

8.2.4 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY

The protocol for [^{125}I]α-BGT was based on that used by Whiteaker et al., (1999) and has been described in detail under General Methodology, *2.3 [^{125}I]α-Bungarotoxin Autoradiography.*

8.2.5 QUANTITATION

Images generated through the *in situ* hybridisation histochemistry and binding protocols were digitally captured and selected anatomical regions were quantified using Scion imaging software. For mRNA images, and [^{125}I]α-BGT autoradiographs the system was calibrated against a set of graded greyscales (Kodak), allowing for quantitation in terms of ROD. For [^{3}H]epibatidine autoradiographs, the system was calibrated against a set of standard tritium scales, allowing for quantitation in terms of nCi/mm^2. The mean ROD or nCi/mm^2 measurements for the vehicle treated groups was compared to the mean ROD or nCi/mm^2 for the mis-ODN and APP-ODN treatment groups using a one-way ANOVA with Dunnett's correction for multiple comparisons. Statistical significance was signified by P values of less then 0.5. The value in all treatment groups represents the mean of measurements taken from 4 animals. The symbol, '*', has been used to denote statistically significant differences between a particular treatment group and the vehicle treated group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Issues relating to quantitation have been described in greater detail in Chapter 2, General Methodology, *2.5 Quantitation.*

8.3 RESULTS

8.3.1 mRNA EXPRESSION OF APP695

The level of APP695 mRNA expression was assessed in coronal slices from the brains of rats treated with a continuous 7-day i.c.v. infusion of either vehicle, mis-ODN or APP-ODN, using *in situ* hybridisation histochemistry (fig. 8.0). Rats treated with the APP antisense ODN probe exhibited a modest but significant increase in APP695 mRNA expression in the cortex (fig. 8.1). This effect was not seen in rats treated with the mis-sense probe.

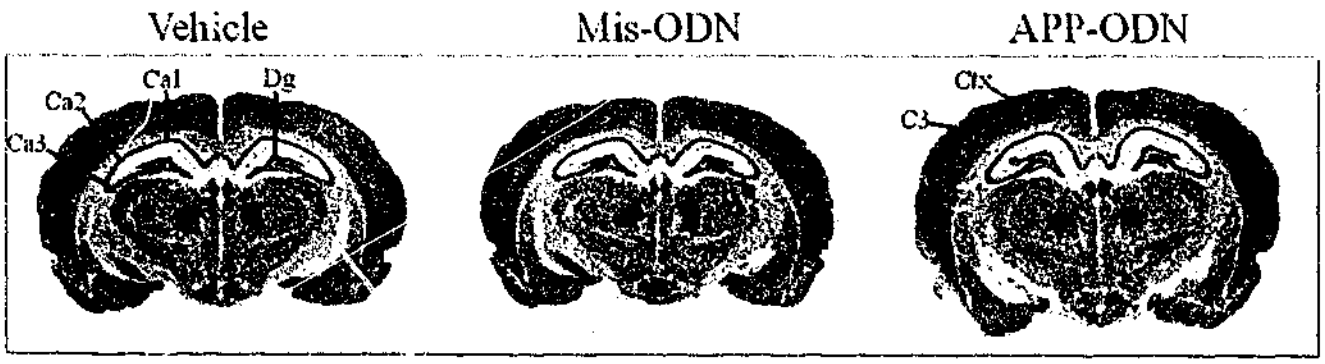


Figure 8.0 Distribution of APP695 mRNA in representative coronal slices taken approximately 3.6 mm caudal to bregma from rats that received a continuous 7-day i.c.v. infusion of either vehicle, mis-ODN or APP-ODN. Abbreviations: APP-ODN, APP695 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C3, cortical layer 3; Ctx, cortex; Dg, dentate gyrus; mis-ODN, mis-sense oligodeoxyribonucleotide. Cortical layers defined as described in the atlas of Paxinos of Watson (Paxinos et al., 1986).

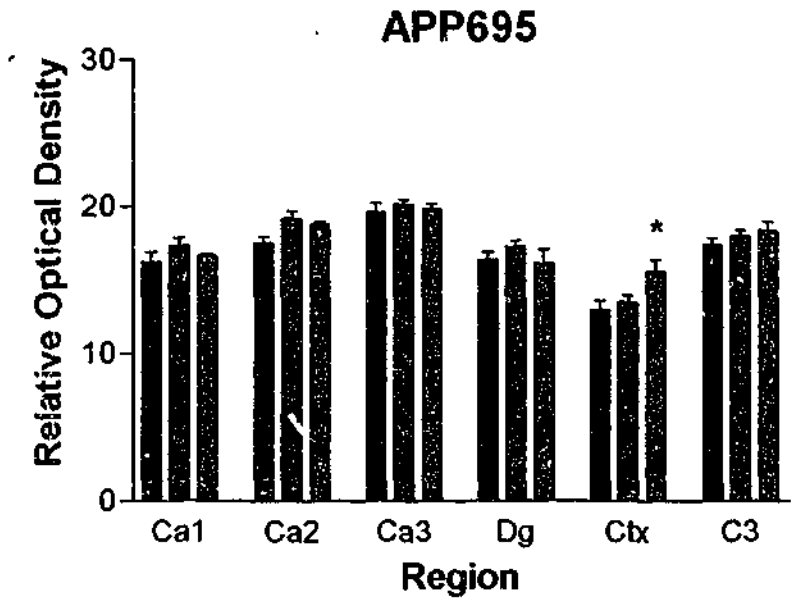


Figure 8.1. Levels of APP695 mRNA, quantified in terms of relative optical density (ROD), in telencephic regions of rats that received a continuous 7-day infusion of either vehicle, mis-ODN or APP-ODN. Abbreviations: APP-ODN, APP695 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C3, cortical layer 3; Ctx, cortex; Dg, dentate gyrus; mis-ODN, mis-sense oligodeoxyribonucleotide. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD for vehicle treated groups was compared to the mean ROD for the α_7ODN and mis-ODN treated groups using one-way ANOVA with a Dunnett post-test (*, $P < 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

8.3.2 nAChR SUBUNIT mRNA EXPRESSION

The effect of inhibiting the translation of APP695 mRNA on the mRNA expression of various nAChR subunits was examined using *in situ* hybridisation histochemistry. The distribution of the α_3, α_4, α_5, α_6, α_7, β_2 and β_3 nAChR subunits in representative coronal sections is shown in figure 8.2.

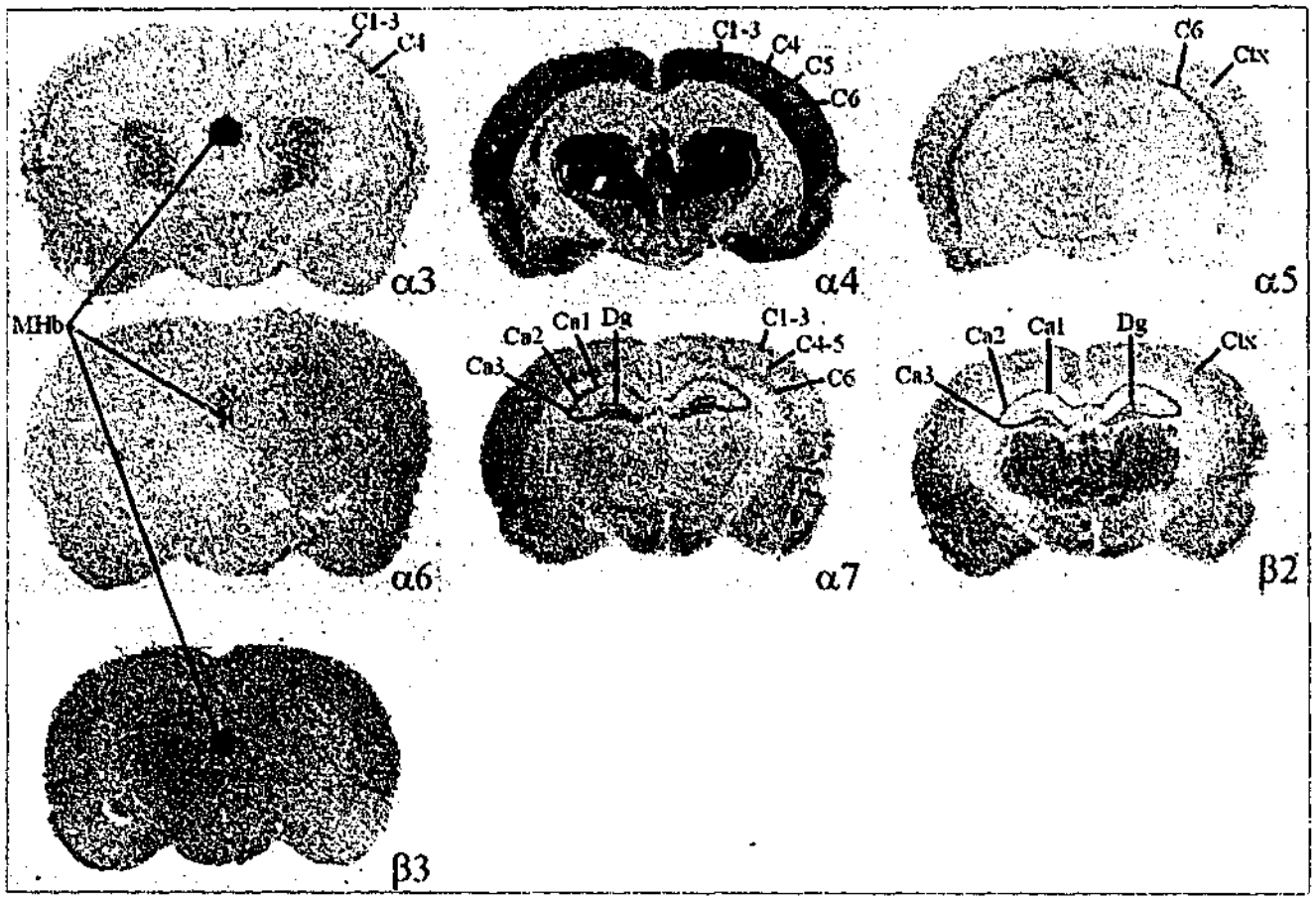


Figure 8.2. Distribution of nAChR subunit mRNA expression in representative coronal sections approximately 3.6 mm caudal to bregma from rats that received a continuous 7-day i.c.v. infusion of either vehicle, mis-ODN or APP-ODN. Abbreviations: Ca1-3, fields of the hippocampus; C1-2, cortical layers 1 – 2; C1-3, cortical layers 1 – 3; C3, cortical layer 3; C4-5, cortical layers 4 – 5; C5-6, cortical layers 5 – 6; C6, cortical layer 6; Ctx, cortex; Dg, dentate gyrus; MHb, medial habenula. Cortical layers defined as described in the atlas of Paxinos and Watson (1986).

Densiometric analysis revealed that i.c.v. infusions of either APP-ODN or mis-ODN did not significantly affect the level of expression of the α_3, α_4, α_5, α_6, α_7, β_2 or β_3 nAChR subunit mRNA relative to vehicle treated rats.

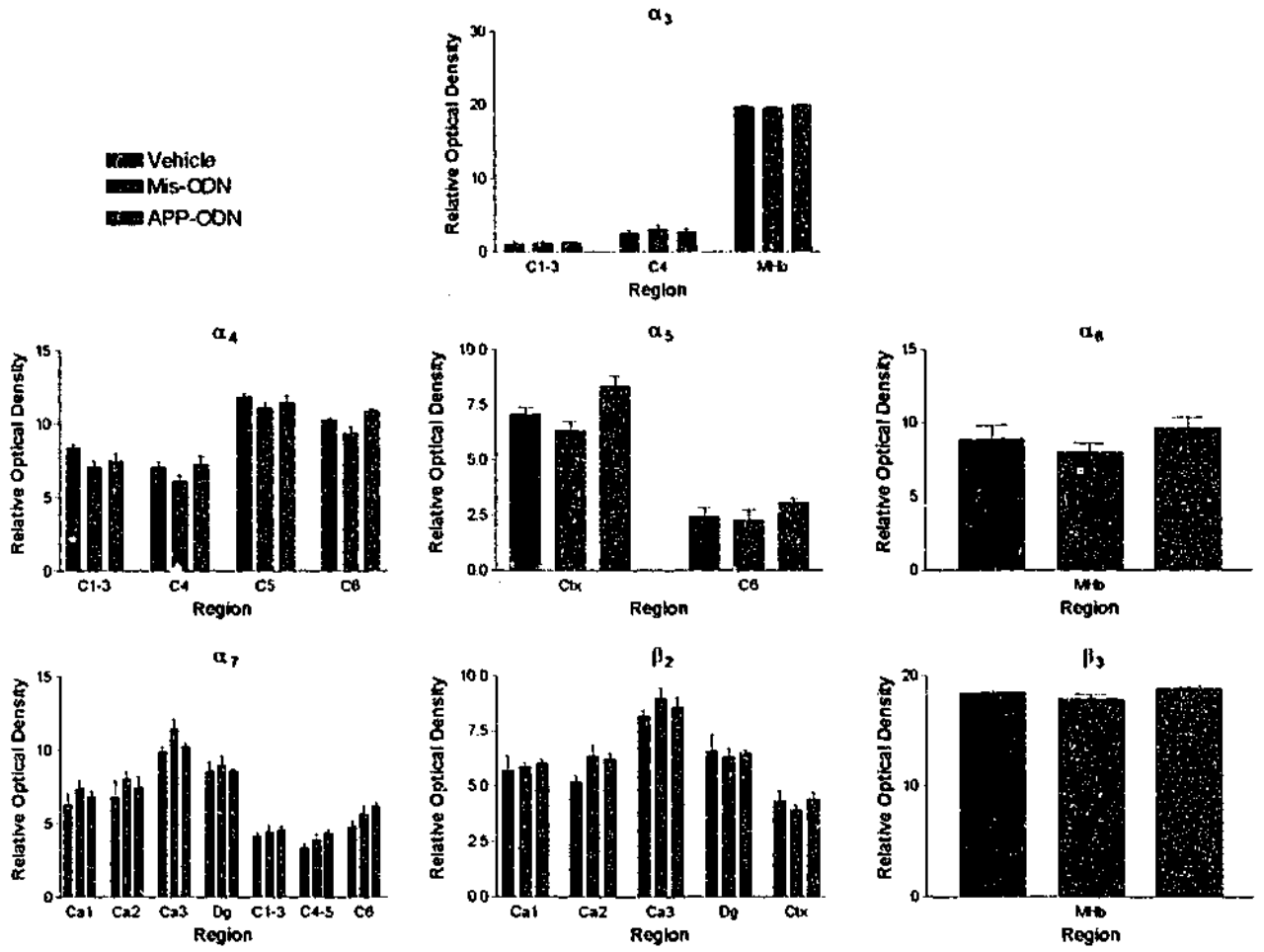


Figure 8.3. Levels of nAChR subunit mRNA, quantified in terms of relative optical density (ROD), in telencephic regions of rats that received a continuous 7-day infusion of either vehicle, mis-ODN or APP-ODN. Abbreviations: APP-ODN, APP695 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C1-2, cortical layers 1 – 2; C1-3, cortical layers 1 – 3; C3, cortical layer 3; C4-5, cortical layers 4 – 5; C5-6, cortical layers 5 – 6; C6, cortical layer 6; Dg, dentate gyrus; mis-ODN, mis-sense oligodeoxyribonucleotide; MHb, medial habenula. Cortical layers defined as described in the atlas of Paxinos and Watson (1986). The mean ROD for vehicle treated groups was compared to the mean ROD for the APP-ODN and mis-ODN treated groups using one-way ANOVA with a Dunnett post-test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

8.3.3 [^{3}H]Epibatidine Autoradiography

The level of α-BGT insensitive or 'non-α_7' nAChRs was measured by analysing [^{3}H]epibatidine autoradiography in each treatment group. The distribution of [^{3}H]epibatidine binding, which reflects predominately the presence of the $\alpha_4\beta_2$ nAChR subtype, is shown in a representative coronal section in figure 8.4.

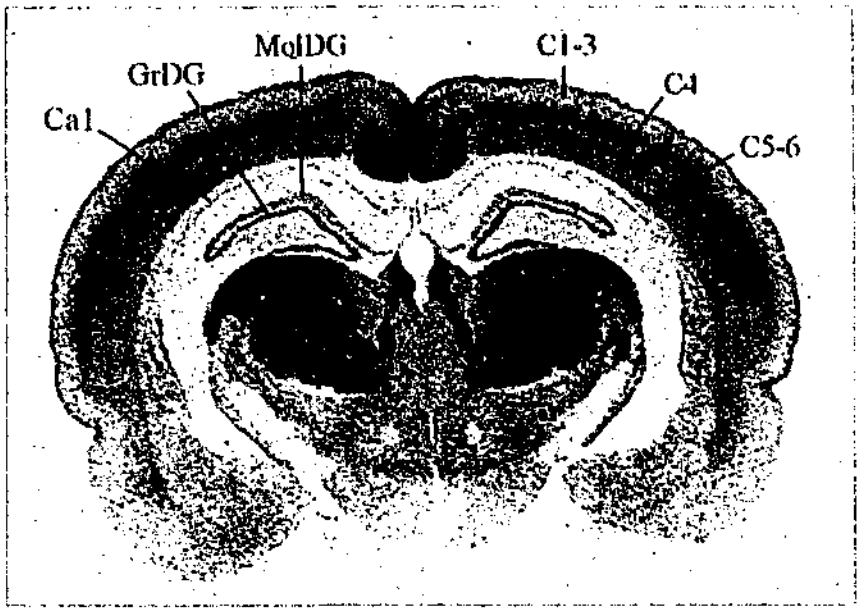


Figure 8.4. Total [^{3}H]epibatidine binding in a representative coronal section approximately 3.6 mm caudal to bregma. Abbreviations: Ca1, field of the hippocampus; C1-3, cortical layers 1 – 3; C4, cortical layer 4; C5-6, cortical layers 5 – 6; GrDG, dentate gyrus granular layer; MolDG, dentate gyrus molecular layer. Cortical layers defined as described in the atlas of Paxinos of Watson (Paxinos et al., 1986).

Densiometric assessment of the autoradiographic images from each treatment group revealed no significant change in the cortical level of [^{3}H]epibatidine binding in rats treated with the APP-ODN relative to vehicle infused rats (fig 8.5). The APP-ODN group exhibited a small reduction of 18% and 22% in cortical layer 4 and the granular layer of the dentate gyrus, however this was not a statistically significant effect. Infusion of the mis-ODN did not significantly affect [^{3}H]epibatidine binding in any of the regions examined.

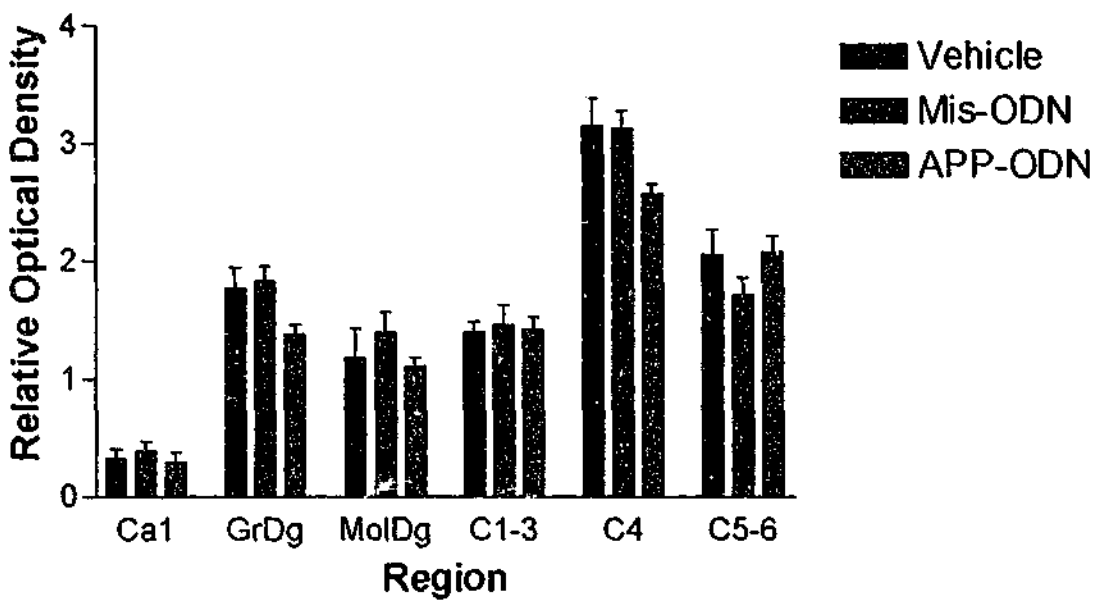


Figure 8.5. Specific [^{3}H]Epibatidine binding, quantified in terms of relative optical density (ROD), in selected cortical and hippocampal regions from rats that received a continuous 7-day infusion of either vehicle, mis-ODN or APP-ODN. Abbreviations: APP-ODN, APP695 antisense oligodeoxyribonucleotide; Ca1, field of the hippocampus; C1-3, cortical layers 1 – 3; C4, cortical layer 4; C5-6, cortical layers 5 – 6; GrDG, dentate gyrus granular layer; mis-ODN, mis-sense oligodeoxyribonucleotide; MolDG, dentate gyrus molecular layer. Cortical layers defined as described in the atlas of Paxinos of Watson (Paxinos et al., 1986). The mean ROD for vehicle treated groups was compared to the mean ROD for the APP-ODN and mis-ODN treated groups using one-way ANOVA with a Dunnett post-test ($P > 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

8.3.4 [^{125}I]α-BUNGAROTOXIN AUTORADIOGRAPHY

The distribution and density of α_7 nAChRs in various cortical and hippocampal regions from rats receiving i.c.v. infusions of either vehicle, mis-ODN or APP-ODN was assessed by examining [^{125}I]α-BGT binding in coronal slices from each treatment group (fig. 8.6).

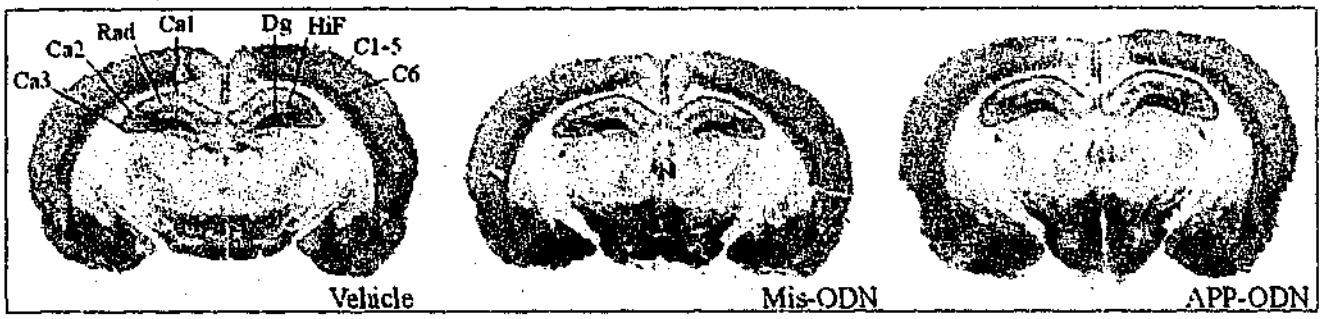


Figure 8.6. Total [^{125}I]α-BGT binding in representative coronal sections approximately 3.6 mm caudal to bregma from rats that received a 7-day continuous i.c.v. infusion of either vehicle, APP-ODN or mis-ODN. Abbreviations: APP-ODN, APP695 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; mis-ODN, mis-sense oligodeoxyribonucleotide; Rad, stratum radiatum hippocampus. Cortical layers defined as described in the atlas of Paxinos of Watson (Paxinos et al., 1986).

The level of specific [^{125}I]α-BGT binding was significantly reduced by 22% in the dentate gyrus of APP-ODN treated rats relative to vehicle infused controls but was not affected in any of the other regions analysed (fig 8.7). The mis-ODN did not significantly affect the level of [^{125}I]α-BGT binding.

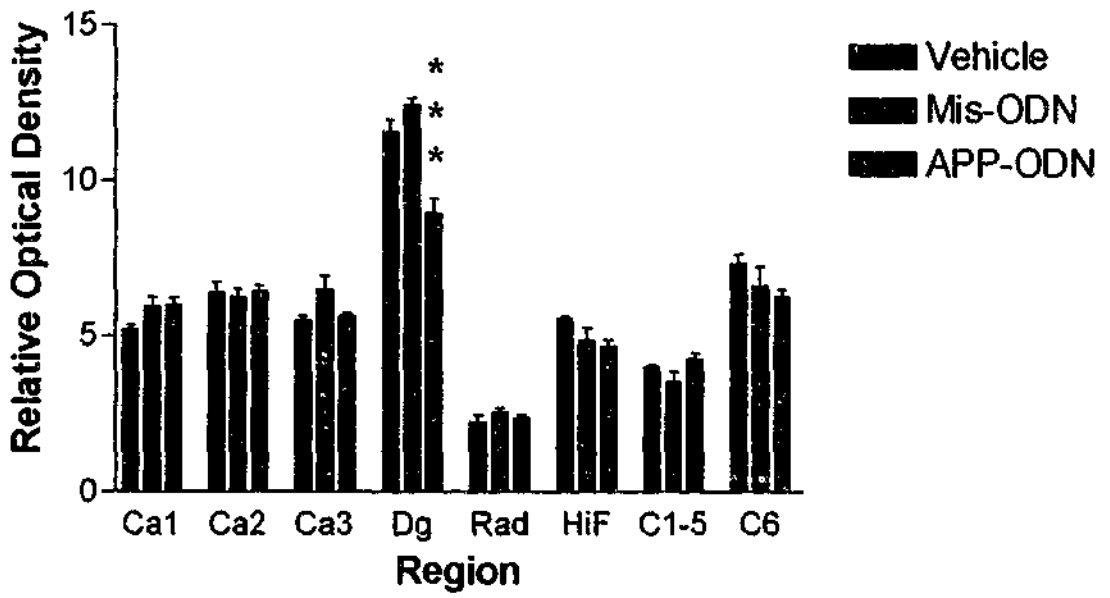


Figure 8.7. Specific [^{125}I]α-BGT, quantified in terms of relative optical density (ROD), binding in rats that received a 7-day continuous i.c.v. infusion of either vehicle, APP-ODN or mis-sense oligonucleotide. Abbreviations: APP-ODN, APP695 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C1-5, cortical layers 1-5; C6, cortical layer 6; Dg, dentate gyrus; HiF, hippocampal fissure; mis-ODN, mis-sense oligodeoxyribonucleotide; Rad, stratum radiatum hippocampus; ROD, relative optical density. Cortical layers defined as described in the atlas of Paxinos of Watson (Paxinos et al., 1986). The mean ROD for vehicle treated groups was compared to the mean ROD for the APP-ODN and mis-ODN treated groups using one-way ANOVA with a Dunnett post-test (***, $P < 0.001$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

8.3.5 mRNA EXPRESSION OF BACE, PS1, PS2 AND TACE

The mRNA expression of a number of other proteins involved in the proteolytic processing of APP, including BACE, the presenilins and TACE, was examined in the three treatment groups using *in situ* hybridisation histochemistry. The distribution of these mRNAs in representative coronal sections from vehicle, APP-ODN and mis-ODN treated rats is shown in figure 8.8.

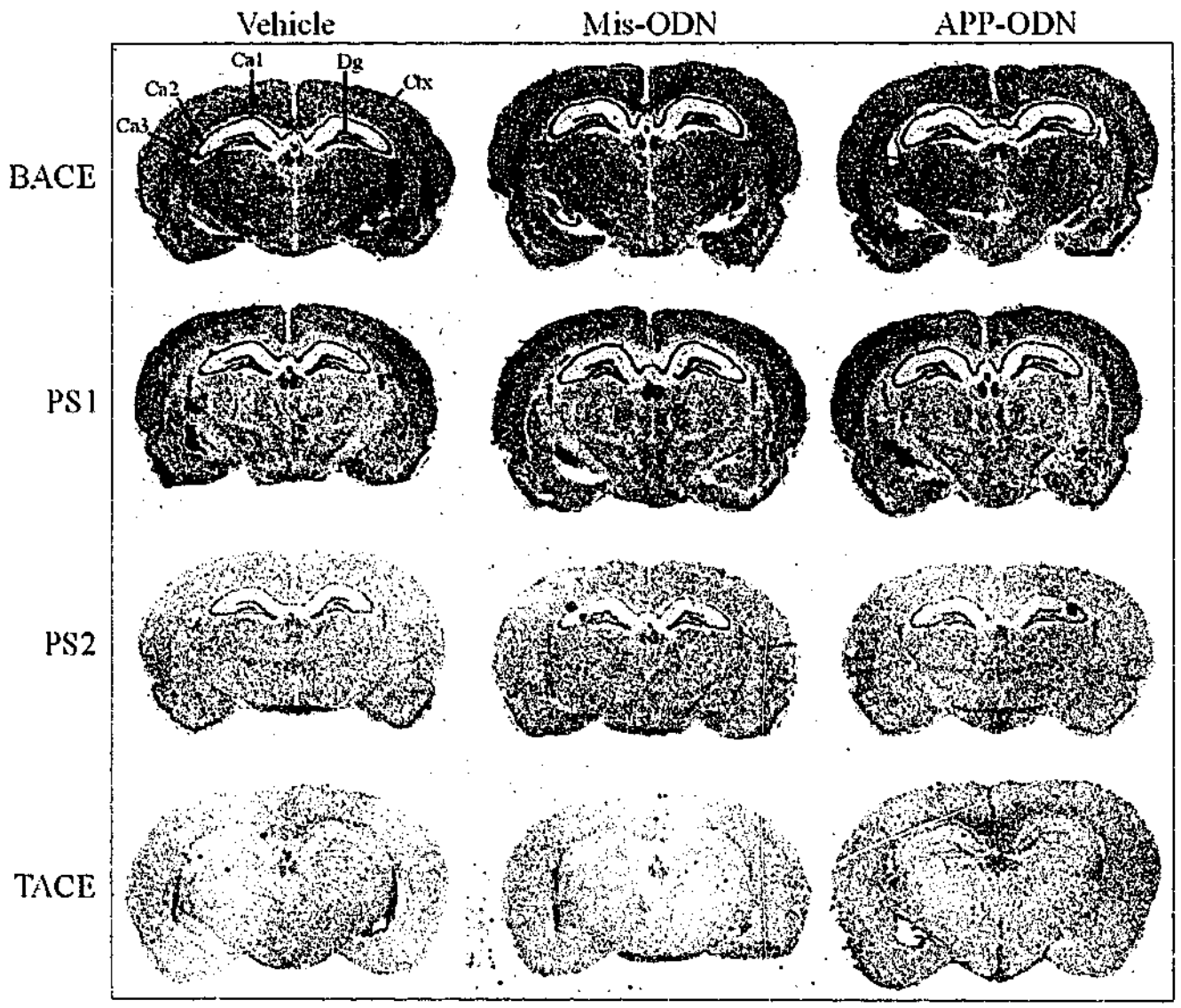


Figure 8.8. Distribution of APP695, β-site APP-cleaving enzyme (BACE), presenilin-1 (PS1), presenilin-2 (PS2) and tumour necrosis factor-α-converting enzyme (TACE) mRNA in representative coronal sections taken approximately 3.6 mm caudal to bregma from rats that received a continuous 7-day infusion of either vehicle, APP-ODN or mis-ODN. Abbreviations:APP-ODN, APP695 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C3, cortical layer 3; Ctx, cortex; Dg, dentate gyrus; mis-ODN, mis-sense oligodeoxyribonucleotide. Cortical layers defined as described in the atlas of Paxinos of Watson (Paxinos et al., 1986).

Continuous i.c.v. infusion of the mis-ODN probe did not significantly affect the level of BACE, PS1, PS2 or TACE mRNA expression in the hippocampus and cortex relative to the vehicle treated group. However, rats treated with the APP antisense probe exhibited a significant increase of 29 – 40% in TACE expression in the cortex and the Ca1, Ca2 and dentate gyrus regions of the hippocampus (fig 8.9). Relatively little TACE mRNA was detected in the Ca3 field of the hippocampus and this was not affected by the APP-ODN treatment. The level of BACE, PS1 and PS2 mRNA expression was not significantly affected by i.c.v. infusions of APP-ODN in the cortical or hippocampal regions examined.

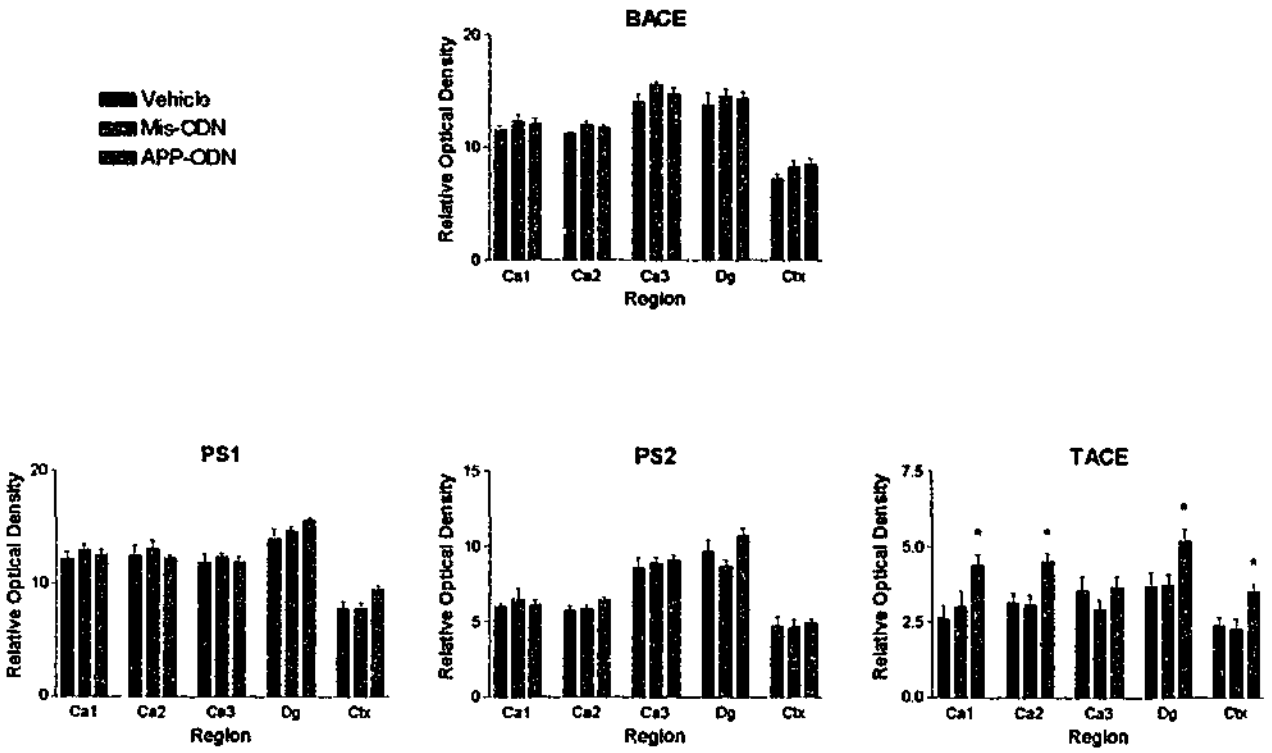


Figure 8.9. Levels of APP695, β-site APP-cleaving enzyme (BACE), presenilin-1 (PS1), presenilin-2 (PS2) and tumour necrosis factor-α-converting enzyme (TACE) mRNA, quantified in terms of relative optical density (ROD), in telencephic regions of rats that received a continuous 7-day infusion of either vehicle, APP-ODN or mis-ODN. Abbreviations: APP-ODN, APP695 antisense oligodeoxyribonucleotide; Ca1-3, fields of the hippocampus; C3, cortical layer 3; Ctx, cortex; Dg, dentate gyrus; mis-ODN, mis-sense oligodeoxyribonucleotide. Cortical layers defined as described in the atlas of Paxinos of Watson (Paxinos et al., 1986). The mean ROD for vehicle treated groups was compared to the mean ROD for the APP-ODN and mis-ODN treated groups using one-way ANOVA with a Dunnett post-test (*, $P < 0.05$). Each data point represents the mean ± S.E.M. of measurements taken from four animals.

8.4 DISCUSSION

As a result of continuous i.c.v. infusion of the antisense ODN probe targeted to APP, there was relatively little change in the level of APP695 mRNA expression, with the exception of a small increase in the expression of APP695 in the cortex which was not localised to specific cortical layers. As discussed in the previous chapter, quantification of the target mRNA will not necessarily be affected by *in vivo* treatment with the antisense ODN, provided that the antisense probe does not hybridise with the target mRNA at the same position as the oligonucleotide probe(s) used for the detection of that mRNA using *in situ* hybridisation histochemstry. The target regions on the complete APP mRNA sequence for the antisense APP-ODN used for i.c.v. infusions and the ODN used for *in situ* hybridisation histochemstry are separated by over 650 base pairs. The findings presented here with regard to APP mRNA expression are consistent with those previously reported by Coulson et al., (1997) which show that i.c.v. injections of antisense ODNs targeted to APP does not affect the level of expression of APP mRNA in a number of brain regions including the amygdala, septum and hippocampus. The small but significant up-regulation of APP mRNA in the cortex reported here may represent a homeostatic response to reduced synthesis of APP and is indicative in ongoing, and perhaps up-regulated, transcriptional activity in response to reduced translation of the mRNA. This has also been suggested by Landgraf et al., (1995) in a study showing that cerebral injections of antisense ODN targeted to the V1 vasopressin receptor subtype results in significantly increased expression of V1 mRNA. A number of studies have shown that a significant increase in APP mRNA expression is also associated with neuronal damage (Nakamura et al., 1992; Ohyagi et al., 1993; Schubert et al., 1993). However, i.c.v. infusion of the mis-sense ODN was not associated with any change in APP mRNA levels and therefore the increase in APP mRNA in the APP-ODN group is not likely to be related to non-specific neuronal

damage caused by ODN infusion or the surgical procedure. Although APP protein levels were not quantified as part of the present study, treatment with similar amounts of antisense APP-ODNs, both *in vitro* and *in vivo*, has consistently been shown to significantly reduce the level of APP protein in a number of other studies (Coulson et al., 1997; Denman et al., 1997; Kumar et al., 2000). The study by Coulson et al., (1997) reports that i.c.v. injections of an APP-ODN does not significantly affect APP mRNA levels, but causes a significant reduction of 43 – 68% in cerebral levels of the APP protein.

Relative to the vehicle treated group, rats treated with the antisense APP-ODN exhibited a significant reduction in [^{125}I]α-BGT binding in the granular layer of the dentate gyrus. This result is indicative of a reduction in density of the α_7 nAChR subtype related to restricted synthesis of the APP protein. Along with the results reported in the previous chapter describing the effects on α_7ODN on the expression of various proteins involved in the proteolytic processing of APP, this finding further supports the hypothesis that there is some sort of interaction between α_7 nAChRs and APP. The level of α_7 mRNA expression was not affected in any of the areas quantified in this study and therefore the reduction in [^{125}I]α-BGT binding is likely to be due to post-transcriptional or post-translational mechanisms rather then a change in transcriptional activity. The apparent discordance between nAChR mRNA expression and receptor protein levels has been well documented (Blumenthal et al., 1997; Ke et al., 1998; Marks et al., 1992; Pauly et al., 1996; Zhang et al., 2000) and has led these authors to conclude that nAChR levels are regulated primarily by post-transcriptional/translational mechanisms. Based on the finding that secreted forms of APP are likely to play an important role in terms of stabilising $[Ca^{2+}]_i$ and therefore preventing excessive neuronal excitability (Li et al., 2000; Mattson et al., 1993b; Mattson et al., 1993a), a sustained inhibition of the synthesis of APP may have significant consequences for $[Ca^{2+}]_i$ in certain neuronal populations. Furthermore, considering the high calcium permeability of the α_7 nAChR, neurons which express α_7 nAChRs may be

particularly vulnerable to such changes which favour increased $[Ca^{2+}]_i$. The dentate gyrus shows one of the highest levels of nAChR density of any area in the brain, which may explain the selective change in α_7 nAChR density in this area. Hypothetically, down-regulation of α_7 nAChR levels in the dentate gyrus as a result of APP-ODN treatment may represent a homeostatic mechanism that is initiated in an attempt to limit α_7 mediated increases in $[Ca^{2+}]_i$, following sustained increase in intraneuronal calcium levels due to lower ambient levels of sAPPs. An important implication for this hypothesis of course is that, to some extent, $[Ca^{2+}]_i$ regulates α_7 nAChR levels. Interestingly, a study by Ospina et al., (1998) has reported that the affinity of agonist binding to α_7 nAChRs is significantly enhanced by the presence of calcium in autoradiographic studies. Furthermore, the α_7 nAChR subtype is rapidly desensitised (Buhler et al., 2001; Fabian-Fine et al., 2001; Mike et al., 2000; Papke et al., 2000) and probably internalised (Fabian-Fine et al., 2001) following agonist binding, and therefore a sustained increase in agonist affinity due to increased calcium levels may also be expected to be associated with an increased level of receptor desensitisation and subsequent internalisation. Of course this is a highly speculative line of reasoning, but offers one possible explanation for the APP-ODN mediated reduction in $[^{125}I]\alpha$-BGT binding reported here. As a useful direction for further work in this area, it would be interesting to investigate the possibility that intraneuronal calcium levels may facilitate an auto-inhibitory feedback loop for α_7 nAChRs.

In terms of the relevance of these results to AD, it has previously been reported that the level of both α_7 nAChR subunits (Burghaus et al., 2000; Guan et al., 2000; Wevers et al., 1999) and secreted forms of APP (Lannfelt et al., 1995; Van Nostrand et al., 1992) are significantly reduced in the brains of AD subjects. The findings reported here indicate that these changes may indeed be related. Specifically, APP may regulate α_7 nAChR levels in certain areas of the brain such as the hippocampus. As has been outlined in previous chapters, the α_7 nAChR has neuroprotective and neurotrophic activities and is thought to

play a critical role in learning and memory function in the hippocampus. Therefore, a loss in the density of these receptors resulting from reduced APP levels may be an important factor in relation to the neurodegenerative changes and clinical symptoms associated with AD. In addition to the development of γ– and β–secretase inhibitors that reduce Aβ formation, an alternative therapeutic strategy for the treatment of AD may involve the development of drugs that increase the level of secreted forms of APP in the brain. The α–secretase enzyme represents a potential target in this context, in that stimulation of α–secretase activity will increase the release of sAPPα and preclude Aβ formation by cleaving APP within the Aβ sequence.

The processing of APP via the α–secretase pathway occurs on the C-terminal side of residue 16 of the Aβ sequence, liberating a soluble N-terminal fragment (sAPPα) from the cell surface and leaving an 83 residue C-terminal fragment anchored to the cell membrane (Esch et al., 1990). Cleavage of APP by α–secretase has both regulated and constitutive components. Regulated α–secretase activity appears to be under the control of protein kinase C (Hung et al., 1993; Sinha et al., 1999b). Although the exact identity of the α–secretase enzyme is not known, there is good evidence to suggest that tumour necrosis factor-α-converting enzyme (TACE) is involved. For example, the inhibition or knockout of TACE significantly decreases the level of α–secretase mediated processing of APP and therefore the release of the sAPPα fragment (Buxbaum et al., 1998). It is reported here that continuous i.c.v. infusion of an antisense ODN probe targeted to the APP695 isoform of APP results in a significant up-regulation in the expression of TACE mRNA in the cortex and hippocampus. This finding further supports existing evidence that TACE is involved in the processing of APP. Furthermore, these results constitute preliminary evidence that TACE activity is regulated by APP levels. As discussed above, although a definitive description of the physiological role for APP in the brain is still lacking, there is a considerable amount of evidence that implicates the proteolytic product, sAPPα, in

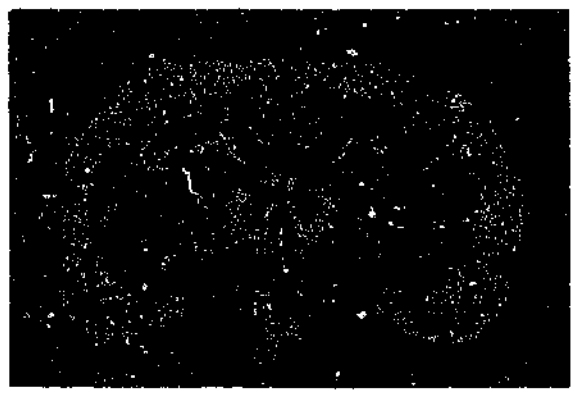
neuronal growth and neuroprotection. Inhibiting the synthesis of APP might be expected to have physiologically relevant consequences by also reducing the level of sAPPα production. One interpretation of the results presented here is that the increase in TACE mRNA expression following transient inhibition of APP synthesis reflects a homeostatic mechanism directed at maintaining a certain level of sAPPα production. Of course an important assumption for such a hypothesis is that increased TACE mRNA expression is translated into increased TACE activity. Further studies that measure TACE protein levels and both constitutive and stimulated α–secretase activity, following APP-ODN treatment, would certainly be justified. It is notable that the expression of mRNA for BACE, which is involved in β-secretase mediated processing, and PS1 and PS2, which are involved in the γ-secretase processing of APP, was not affected by APP-ODN treatment. Therefore, inhibition of APP synthesis may specifically increases α–secretase processing of APP and the production of sAPPα, rather than non-specifically up-regulating processing at the α–, β– and γ–secretase sites and the production of other APP fragments such as sAPPβ, p3 and Aβ. This further supports the notion that sAPPα represents an important and physiologically relevant product of APP processing.

In conclusion, the work presented in this chapter demonstrates that α_7 nAChR receptor levels in the dentate gyrus and TACE mRNA expression in the hippocampus and cortex may be regulated by the level of APP695 or its proteolytic products. Considering the importance of the α_7 nAChR in neurotrophic, neuroprotective and cognitive activity, a loss of this receptor as a result of reduced APP synthesis may be an important factor in the neurodegeneration and clinical symptoms associated with AD. The finding that TACE mRNA expression is significantly increased in APP-ODN treated rats indicates that the brain may possess the capacity to maintain a certain level of sAPPα through changes in α–secretase activity when the availability of the APP substrate is limited. Stimulation of the α–secretase mediated processing of APP may represent a useful therapeutic approach

in the treatment of AD. Furthermore, this approach may offer advantages over the use of β– and γ–secretase inhibitors if one considers that increased α-secretase activity would achieve the same goal, in terms of reducing Aβ formation, but also increase the production of the neurotrophic/neuroprotective sAPPα molecule and not interfere with normal cellular processes that may rely on β– and γ–secretase processing of APP.

Chapter 9

General Discussion



Alzheimer's disease is the major cause of the severe intellectual decline described as dementia and is thought to affect over half of the population over the age of 85 in industrialised nations (Hof et al., 1995). This presents a substantial financial and, of course, emotional cost to the community. Consequently, AD has been the subject of more scientific research than any other disorder of the central nervous system. Despite an enormous amount of time and money dedicated to the study of AD, surprisingly little is known about the precise nature of the events that precipitate the neurodegenerative processes associated with the disease. In order to gain a better understanding of these neurodegenerative processes and identify which pathological changes represent the best targets for therapy, it would be particularly useful to establish 'cause and effect' relationships between the vast array of neurochemical changes seen in AD brains. In this context, the central aim of the work presented here has been to examine a number of the neurochemical markers thought to play important roles in AD pathology, and to explore possible relationships between changes in these neurochemical markers based on rodent models of specific pathological changes associated with AD. Although the results presented throughout this thesis have been discussed in some detail at the end of each chapter, it is worthwhile briefly discussing some of the more interesting results that relate across multiple chapters, and their relevance to AD. It may be useful to briefly review some of the major findings and conclusions presented in each chapter first.

CHAPTER 3:

- A continuous 14-day i.c.v. infusion of $A\beta_{(1-40)}$ causes a significant reduction in the binding of [^{3}H]HC-3 to high affinity choline uptake sites in the cortex and hippocampus, but does not affect ChAT activity or AChE levels. It was concluded from this finding that chronic elevation of $A\beta_{(1-40)}$ levels in the brain may modulate the normal functioning of cholinergic independently of a direct neurotoxic effect.

- Chronic nicotine treatment at doses similar to those which may be experienced as a result of smoking also caused a significant reduction in cortical and hippocampal [^{3}H]HC-3 binding. Furthermore, chronic nicotine treatment significantly

potentiated the reduction in [^{3}H]HC-3 binding caused by $A\beta_{(1-40)}$ infusions. One interpretation of this finding is that chronic nicotine exposure associated with smoking may exacerbate the neuromodulatory and potentially pathological effects mediated by elevated Aβ levels in the brain.

- Chronic nicotine treatment at a dose of either 0.75 or 1.25 mg/kg/day resulted in a significant up-regulation of [^{3}H]epibatidine binding to non-α_7 nAChR subtypes in the hippocampus and cortex. This affect was somewhat more pronounced in rats also treated with $A\beta_{(1-40)}$. Binding of [^{125}I]α-BGT to α_7 nAChRs was not affected by chronic nicotine treatment, continuous i.c.v. $A\beta_{(1-40)}$ infusion or a combination of these treatments.

- The mRNA expression of the α_4, β_2 or α_7 nAChR subunits was not significantly affected by either chronic nicotine or $A\beta_{(1-40)}$ treatment. However, a combination of chronic nicotine and $A\beta_{(1-40)}$ treatment results in a significant increase in α_7 mRNA levels in the Ca1 field of the hippocampus.

- Chronic nicotine treatment at a dose of 1.25 mg/kg/day caused a significant reduction in both NR1 and NR2A, but not NR2B, NMDAR subunit mRNA in the cortex and Ca3 field of the hippocampus. This effect is abolished by co-treatment with i.c.v. infusion of $A\beta_{(1-40)}$.

- Expression of APP695, BACE, PS1, PS2 or TACE mRNA was not significantly affected by chronic nicotine treatment, continuous i.c.v. $A\beta_{(1-40)}$ infusion or a combination of these treatments.

CHAPTER 4:

- The i.c.v. injection of 192-IgG-saporin caused the selective degeneration of cholinergic basal forebrain neurons as demonstrated by a significant reduction in AChE levels in areas that receive basal forebrain innervation. The significant recovery in AChE levels as early as 7 days after 192-IgG-saporin treatment indicates some degree of plasticity associated with the cholinergic basal forebrain system.

- 192-IgG-saporin treatment caused a modest but significant increase in the level of α_4 and α_7 nAChR subunit mRNA in the cortex and hippocampus respectively. This effect was only seen at later time points following i.c.v. 192-IgG-saporin injection. The time dependent nature of this effect may relate to the regeneration rather than degeneration of cholinergic basal forebrain neurons. The level of β_2 subunit mRNA was not affected by 192-IgG-saporin treatment.

- The binding of [^{125}I]α-BGT and [^{3}H]epibatidine to α_7 and non-α_7 nAChR subtypes respectively was not affected as early as 4 days and up to 90 days after i.c.v. 192-IgG-saporin injection.

- The level of NR2A NMDAR subunit mRNA was significantly up-regulated in the cortex and Ca1 field of the hippocampus after the i.c.v. injection of 192-IgG-saporin. Again this effect was time dependent and was only seen 90 days after 192-IgG-saporin treatment. The level of NR1 and NR2B mRNA was not affected and therefore degeneration of the cholinergic basal forebrain neurons may selectively affect certain NMDAR subtypes.

- There was a significant increase in the expression of nNOS mRNA in the dentate gyrus detectable 7 days after 192-IgG-saporin treatment and persisting until 90 days after treatment.

- APP695 mRNA expression was significantly increased by i.c.v. injection of 192-IgG-saporin. This effect was not seen until 90 days after treatment with 192-IgG-saporin. One interpretation of this finding is that the degeneration of the cholinergic forebrain system associated with AD may be a causative factor in the increased production of APP fragments such as Aβ. No change in the level of BACE, PS1, PS2 or TACE mRNA was seen after 192-IgG-saporin treatment. The expression of these proteins is not likely to be under the control of the cholinergic basal forebrain system.

CHAPTER 5:

- Excitotoxic lesioning of the nbM region in the basal forebrain, using quinolinic acid, resulted in significant increases in α_4, β_2 and α_7 nAChR subunit mRNA in the hippocampus and cortex. In light of the relatively small changes in the expression of these subunits associated with 192-IgG-saporin treatment, it was concluded that non-cholinergic basal forebrain neurons (i.e. GABAergic) may regulate the expression of certain nAChR subunits.

- The increase in hippocampal and cortical α_4 and β_2 nAChR was not accompanied by a concomitant increase in [^{3}H]epibatidine binding following excitotoxic nbM lesions. As discussed in chapter 3, this finding may reflect an up-regulation of $\alpha_4\beta_2$ nAChRs in the hippocampus and cortex, masked by a loss of presynaptic $\alpha_4\beta_2$ nAChRs on basal forebrain afferents. The significant increase in [^{125}I]α-BGT binding in the same regions which showed increased α_7 mRNA expression following excitotoxic nbM lesions was interpreted as an increase in α_7 nAChR density mediated at the transcriptional level.

- Injection of quinolinic acid into the nbM resulted in a much greater response in the level of expression of hippocampal and cortical NMDAR subunit mRNA relative to the modest increase in NR2A mRNA expression following i.c.v. 192-IgG-saporin injection. Specifically, excitotoxic nbM lesions cased a significant increase in NR1 and NR2B subunit mRNA in the cortex and hippocampus while NR2A mRNA was unaffected. It was concluded that non-cholinergic basal forebrain neurons may play an important role in reguiating specific NMDAR populations in these regions.

- There was a significant increase in cortical and hippocampal APP695 expression following excitotoxic basal forebrain lesions, which was more pronounced than that associated with 192-IgG-saporin treatment. Both cholinergic and non-cholinergic basal forebrain neurons may play a role in regulating APP695 levels. Unlike 192-IgG-saporin treatment, quinolinic acid injections caused a significant increase in mRNAs corresponding to proteins involved in the non-amyloidogenic processing of APP, including PS1, PS2 and TACE.

CHAPTER 6:

- Binding of [^{125}I]α-BGT to α_7 nAChRs in hippocampal membrane fragments was readily displaceable by the selective α_7 nAChR agonist, choline, but not by the $A\beta_{(12\text{-}28)}$ or $A\beta_{(1\text{-}42)}$ peptides.

- Binding of [^{125}I]α-BGT to α_7 nAChRs in the whole hippocampal slice was readily displaceable by the selective α_7 nAChR agonists, MLA and choline, but not by the $A\beta_{(12\text{-}28)}$ or $A\beta_{(1\text{-}42)}$ peptides.

- The continuous 14 day i.c.v. infusion of $A\beta_{(1\text{-}40)}$ did not significantly affect the expression of α_7 mRNA or binding of [^{125}I]α-BGT to α_7 nAChRs in the hippocampus or cortex. Based on studies which show that nAChRs are up-regulated by chronic treatment with either agonists (Barrantes et al., 1995; Ke et al., 1998) or antagonists (Molinari et al., 1998), it was concluded that the $A\beta_{(1\text{-}40)}$ did not bind to α_7 nAChRs.

- The main conclusion from work presented in chapter 6 was that Aβ peptides do not bind competitively to the α_7 nAChR subtype as has been previously reported (Wang et al., 2000a & 2000b).

CHAPTER 7:

- Transient knockdown of the α_7 nAChR protein by the continuous i.c.v infusion of an antisense ODN probe targeted to α_7 nAChR subunit mRNA caused a significant

reduction in [^{125}I]α-BGT binding throughout the hippocampus and cortex. There was also a more restricted reduction in α_7 mRNA levels. Breakdown of the α_7ODN-mRNA hybrid as well as inhibited ribosomal translation may both be important mechanisms in inhibiting α_7 subunit protein synthesis following i.c.v. α_7ODN infusion.

- There was a significant up-regulation of α_4 and β_3 nAChR subunit mRNA in the dentate gyrus following continuous i.c.v infusion of the α_7ODN probe. This finding may reflect the up-regulation of novel nAChR subtypes in response to a reduction in α_7 nAChR levels.

- The level of [^{3}H]epibatidine binding to non-α_7 nAChRs was significantly reduced in the cortex following α_7ODN treatment.

- Continuous i.c.v. infusion of the α_7ODN caused a significant up-regulation of PS1, PS2 and TACE mRNA in the ventricular zone cells. It was concluded that α_7 nAChR activation may play an important role in regulating APP processing along the non-amyloidogenic α-secretase pathway.

CHAPTER 8:

- A small but significant increase in cortical APP695 mRNA levels was observed as a result of continuous i.c.v infusion of an antisense ODN probe targeted to APP695 mRNA.

- Continuous infusion of APP-ODN caused a significant increase in TACE mRNA levels in the hippocampus and cortex. Reductions in APP levels may stimulate the processing of APP at the α-secretase site.

- There was a significant reduction in binding of [^{125}I]α-BGT to α_7 nAChRs in the dentate gyrus following APP-ODN treatment. The level of α_7 subunit mRNA expression was not affected and therefore APP levels may regulate α_7 nAChR levels through a post-transcriptional mechanism.

Overall, based on these results, it may be concluded that a number of neurochemical markers are readily manipulated by models of specific neurodegenerative and morphological changes associated with AD. Furthermore, evidence has been presented

showing that there may be causal relationships between changes in certain groups of neurochemical markers seen in AD brains. Two such changes, which are often described as being definitive for AD neuropathology, include the selective degeneration of cholinergic basal forebrain neurons and a significant increase in the accumulation of Aβ peptides in deposits throughout the brain. A central focus in AD research has been to establish a causal relationship between cholinergic basal forebrain denervation and increased Aβ levels by addressing the question - "Which change is causative factor relative to the other in the context of neurodegeneration associated with AD?" The work presented here indicates that, to some degree, the answer may be "both". In chapter 3 it was demonstrated that a chronic elevation in the level of $A\beta_{(1\text{-}40)}$ in the brain can modulate the function of cholinergic neurons by reducing the density of high affinity choline uptake sites. In light of evidence that choline deprivation may eventually cause damage to cholinergic neurons (Wurtman, 1992), this finding supports the hypothesis that Aβ accumulation may play a causative role in relation to the degeneration of cholinergic basal forebrain neurons seen in AD. However, results presented in chapter 4 showed that degeneration of the cholinergic basal forebrain neurons results in a significant increase in the expression of predominate APP isoform in the brain, APP695. Based on reports showing that increased expression of APP in rats is also associated with a significant increase in the formation of Aβ peptides (Games et al., 1995), it may be concluded that this finding supports the hypothesis that degeneration of the cholinergic basal forebrain system directly contributes to the accumulation of Aβ deposits. Collectively, the results from chapters 3 and 4 indicate that there may be a reciprocal relationship between cholinergic basal forebrain activity and Aβ levels. An important consequence of this hypothesis is that a change in either of these systems may cause a self-perpetuating pathological response resulting in both cholinergic neuron loss and Aβ accumulation. Clearly maintenance and restoration of the cholinergic

basal forebrain system and prevention of Aβ accumulation may be equally important strategies in the therapy of AD.

One of the central aims of the work presented here was to examine how binding of [^{125}I]α-BGT and [^{3}H]epibatidine to α_7 and non-α_7 nAChR subtypes, as well the expression of nAChR subunit mRNAs, is affected in various experimental paradigms which model certain aspects of AD. A number of findings have been described that relate to possible cause and effect relationships between the neurochemical changes seen in AD brains. For example, in chapter 7 it was demonstrated that transient inhibition of the synthesis of the α_7 nAChR subunit *in vivo* is associated with a significant reduction in the density of the α_7 nAChR subtype in the cortex and hippocampus, but also a significant reduction in the binding of [^{3}H]epibatidine to non-α_7 nAChR subtypes in the cortex. This finding raises the notion that there may be a relationship between the loss of $\alpha_4\beta_2$ nAChRs (Nordberg et al., 1986) and α_7 nAChRs (Wevers et al., 1999) seen in AD brains. The finding that transient inhibition of the synthesis of the APP695 protein causes a significant reduction in α_7 nAChR levels, presented in chapter 8, indicates that there may also be a relationship between the reduced levels of secreted forms of APP found in the CSF of AD patients (Van Nostrand et al., 1992) and a loss of α_7 nAChRs. Collectively, these findings suggest that the α_7 nAChR may play a prominent role in the neuropathological changes associated with AD, and substantiates the idea that the α_7 nAChR represents a potentially important therapeutic target for the treatment of AD (Kem, 2000).

In terms of how the significant loss of nAChR levels associated with AD may be related to specific pathological changes, no evidence was found to indicate that elevated levels of Aβ might directly affect nAChR levels. Furthermore, the degeneration of both cholinergic and non-cholinergic basal forebrain neurons was found to *increase* the expression of certain nAChR subunits as well as binding of [^{125}I]α-BGT and [^{3}H]epibatidine to nAChRs. While this finding may indicate that the loss of nAChR density

seen in AD is not directly related to the degeneration of the basal forebrain, it should be noted that measurement of neurochemical markers in AD brains is usually taken from post-mortem tissue representing end stages of the disease. While the progression of AD neurodegeneration takes years, and even decades, the measurement of neurochemical markers in this study was performed a matter of only weeks after the initial experimental manipulation. Therefore, the up-regulation of nAChRs seen here may represent an early response to degeneration of the basal forebrain structure that precedes an eventual loss of nAChRs associated with latter stages of the disease. The same conclusion might be made for other neurochemical markers such as NMDAR subunits and nNOS, which were increased following excitotoxic or selective cholinergic lesions but have been reported to be significantly down-regulated in the brains of AD subjects (Greenamyre et al., 1985; Thorns et al., 1998). Collectively, these findings may have important implications for the treatment of AD at early stages of the disease. For example, the effect of cholinergic or nicotinic therapy of AD, may vary between late stages of the disease, when there may be a significant amount of cholinergic hypofunction and early stages where, as has been reported here, there may be an up-regulation of certain cholinergic markers, such as nAChRs. Furthermore, the up-regulation of multiple excitatory neurochemical markers, such as nAChRs, NMDARS and nNOS, reported here as an early response to certain neurodegnerative changes associated with AD, suggests that limiting excitotoxic forms of neurodegeneration may be an important strategy in the early treatment of the disease.

Perhaps one of the more interesting findings to come out of the work presented here, details preliminary evidence that chronic nicotine exposure, such as that associated with smoking, may exacerbate the potentially damaging effects in relation to cholinergic neurons associated with elevated Aβ levels. Clearly this finding does not support the epidemiological study by van Duijn et al., (1991) reporting that chronic nicotine intake associated with smoking is positively correlated with a significant increase in the age of

onset for AD. However, a recently published review by Kukull, (2001) has discussed a number of experimental biases associated with this and other studies that report a protective effect of smoking in relation to AD. Furthermore, Kukull, (2001) have described studies that correct for these experimental biases and show a moderate increase in the risk of AD in smokers (Merchant et al., 1999; Ott et al., 1998). While the exact nature of any association between smoking and AD remains controversial, the results presented here support studies showing an increased risk of AD associated with smoking and also describe a possible mechanism for such an interaction involving nicotine, Aβ and high affinity choline uptake sites.

Another group of related neurochemical markers examined throughout this thesis included the neuronal isoform of APP, APP695, as well as a number of proteins involved in the proteolytic processing of APP including BACE, PS1, PS2 and TACE. Evidence has been presented showing that damage to both cholinergic and non-cholinergic basal forebrain neurons may be important factors related to the up-regulation of APP (and therefore perhaps Aβ) levels in the brain. Interestingly, two distinct experimental manipulations resulted in the selective up-regulation of mRNAs corresponding to proteins associated with the processing of APP along the non-amyloidogenic α-secretase pathway. Specifically, both excitotoxic basal forebrain lesions as well as transient knockdown of the α_7 nAChR subunit using an antisense ODN probe caused a significant up-regulation of PS1, PS2 and TACE in various hippocampal and cortical regions. As discussed in chapter 7, it was concluded that because α_7ODN treatment results in a significant reduction in α_7 nAChR levels in these regions, there may be an important relationship between increased mRNA expression of PS1, PS2 and TACE mRNA and a loss of α_7 nAChRs in AD. However, at odds with this conclusion, the excitotoxic basal forebrain lesions that caused a significant *increase* in α_7 nAChR levels, also resulted in an up-regulation of PS1, PS2 and TACE mRNA. Collectively, these results indicate that there may be a specific factor

common to both excitotoxic basal forebrain lesions and transient α_7 nAChR knockdown that regulates the expression of proteins involved in processing of APP along the α-secretase pathway. One may therefore conclude that there may be a close relationship between two important changes associated with AD, a loss of α_7 nAChRs and the degeneration of basal forebrain neurons, and the non-amyloidogenic processing of APP. Further work in this area may involve investigations into specific factors related to the regulation of APP processing, and experimental manipulations that favour processing of APP along the amyloidogenic β-secretase pathway rather than the non-amyloidogenic α-secretase pathway.

It should be stated that while some interesting results have been presented here, as is often the case, the most significant contribution to the field of AD research advanced by this thesis is perhaps not the experimental data itself but rather directions for future work based on this data. Two of the more promising directions for further work relate to the finding that the modulation of certain aspects of cholinergic neuron function by Aβ is potentiated by chronic nicotine treatment, and that transient knockdown of the α_7 nAChR causes the selective up-regulation of mRNA for proteins involved in the a α-secretase processing of APP. Clearly, based on the prevalence of smoking in the community, the finding that chronic nicotine exposure may have specific and detrimental effects in relation to the pathogenesis of AD should not be understated. This finding represents a clear direction for further work in the already intensely studied field of nicotine and nAChR pharmacology in AD. Specifically, the investigation of the exact nature of the interaction between nicotine and Aβ, in relation to the modulation and, potentially, the degeneration of cholinergic neurons. Furthermore, the implication that nicotine may exacerbate degenerative changes associated with elevated Aβ levels goes somewhat against the current thinking in this field, which has produced findings showing a positive correlation between smoking and delayed AD onset (van Duijn et al., 1991) and nicotine mediated

neuroprotection against Aβ toxicity in vitro (for reviews see Shimohama et al., 2001; Zamani et al., 2001). It is not known whether the effects of nicotine in relation to the Aβ mediated change in cholinergic neuron function reported here, and elsewhere (for review see Auld et al., 1998), are related to specific nAChR subtypes. Considering the current focus in AD research on the potential of selective nAChR ligands in the treatment of AD, this is an area that warrants further research. It would be particularly interesting to investigate the effects of chronic treatment with nicotine and selective nAChR ligands, such as GTS-21, in the transgenic rodent model of AD characterised by Games et al., (1995) which over-expresses a mutant form of APP and consequently produces significantly greater amounts of the Aβ peptide.

The finding that mRNAs corresponding to proteins associated with the production of sAPPα from APP (PS1, PS2 and TACE) are significantly up-regulated following transient knockdown of the α_7 nAChR is another result that is worth exploring further. It was particularly interesting to note that the up-regulation in these mRNAs was most prominent in the ventricular zone. The ependymal layer that forms part of the neuroepithelial lining of the ventricles is an important source of neural progenitor cells in the mammalian brain. In chapter 8, following a detailed discussion of the available literature regarding the neurotrophic activities of both sAPPα and α_7 nAChRs and their relevance to neural precursor cells, it was concluded that stimulation of the α-secretase processing of APP and the subsequent production of sAPPα by α_7 nAChRs may represent an important neurotrophic and neurogenic mechanism in the mammalian brain. The use of cell replacement therapy for the treatment of neurodegenerative disease is a promising and rapidly growing field, but one that is limited by the availability of appropriate neuronal material (Bjorklund et al., 2000). A number of researchers are presently investigating the possible use of neural progenitors derived from the ventricular zone and expanded *in vitro* as donors in cell replacement therapy of neurodegenerative disorders such as Parkinson's

disease (Sáwamoto et al., 2001). The expansion and differentiation of neuronal precursor cells both *in vivo* and *in vitro* is critically dependant on extrinsic regional signals (Bjorklund et al., 2000). Based on the results presented in chapter 8 showing a relationship between α_7 nAChR density and the mRNA expression of PS1, PS2 and TACE in the ventricular zone cells, it is worth investigating the possibility that selective α_7 nAChR ligands may be useful tools in the field of cell replacement therapy and neural stem cell research.

The results presented throughout this thesis show that a wide range of neurochemical markers are differentially affected in response to specific neurodegenerative and morphological changes associated with AD. Furthermore, evidence has been presented and discussed showing that, in many cases, changes in these neurochemical markers are likely to be interrelated. This is something that should certainly be considered as part of the development of therapeutic strategies that seek to alter the expression or function specific neurochemical markers for the treatment of AD. In AD, the pathogenesis of the disease is likely to be related to a delicate, and probably complicated, balance between many neurochemical systems, including cholinergic neuron function, APP processing and nAChR pharmacology. The work presented here has shown that manipulation of one of these systems can have important consequences for the functioning of the others. This is consistent with the current line of thinking in AD, in that the pathogenesis of the disease is likely to be multifactorial in nature, involving genetic and environmentally based changes in many biological systems in the brain. Consequently, effective therapy of AD is also likely to involve the treatment of multiple systems. Investigations into possible relationships between changes in certain neurochemical markers and biological systems, and their relevance to the neurodegenerative changes and clinical symptoms associated with AD, will continue to be an important part of the development of multifactorial therapeutic strategies.

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