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Homeostatic and non-homeostatic control of feeding and behaviour

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Abstract

The neural circuits controlling food intake and feeding-related behaviour can be broadly classified as homeostatic or non-homeostatic. Homeostatic circuits respond to the metabolic needs of an organism and drive or supress feeding accordingly. In contrast, non-homoeostatic circuits are able to override metabolic cues to modify feeding and motivation in response to environmental or sensory cues that could be aversive, for instance an immediate threat; or appetitive, such as the hedonic properties of a food.

Historically, research has focused on the homeostatic control of food intake. We now have a relatively comprehensive understanding of the key neuronal populations that respond to peripheral signals of nutrient status. The agouti-related peptide (Agrp) neurons of the hypothalamus are the canonical hunger-sensing neurons, in that they sense changes in circulating metabolites and act to promote food intake and avoid starvation. Recently, a number of studies have reported that the Agrp neurons do not simply promote food consumption, but also act to suppress competing drives to promote adaptive food-seeking behaviours. In chapter two presented herein, we investigate whether Agrp activation in the face of an acute stressor promotes adaptive behavioural and hormonal responses. We use cutting-edge techniques, including chemogenetics and wireless optogenetics to remotely activate Agrp neurons. Our results demonstrate that Agrp activation prior to an acute stressor reduces anxiety, improves memory recall and promotes food intake in a threatening environment. At the same time, Agrp activation potentiates the corticosterone response to stress. We considered whether the Agrp induced hormonal changes were required for the expression of adaptive behaviours. Through inhibition of corticosterone, we demonstrate that the adaptive behavioural and hormonal effects of Agrp activation prior to stress occur independently. We conclude that the Agrp neurons coordinate both behavioural and hormonal responses to acute stress to promote adaptive food-seeking behaviours that would allow a hungry animal to enter unfamiliar and threatening environments in the search for food.

In contrast, the neural circuits responsible for non-homeostatic feeding remain unclear. These circuits become particularly important to consider in conditions of extreme overweight or underweight, such as obesity or anorexia, where feeding behaviour is inconsistent with metabolic need. Human neuroimaging studies implicate both the medial pre-frontal cortex (mPFC) and anterior cingulate cortex (ACC) in both obesity and anorexia, suggesting these regions may be involved in non-homeostatic feeding drive. Classical animal studies implicate the lateral hypothalamus (LH) as a key integrator of top-down information with bottom-up metabolic signals. The LH receives input from a number of cortical regions include the mPFC and ACC. In chapters three and four, we use genetic, chemogenetic and optogenetic tools to investigate the roles of the mPFC-LH circuit and the ACC-LH circuit. Our results demonstrate that both the mPFC-LH circuit and ACC-LH circuit act to suppress food intake. In addition, the mPFC-LH circuit encodes aversion and supresses motivated feeding-behaviours; while the ACC-LH circuit appears to promote locomotor activity. Our results identify novel appetite suppressing cortical to hypothalamic circuits that may be relevant to improving our understanding of conditions such as obesity and anorexia.

Publications during enrolment

Clarke RE, Verdejo-Garcia A, Andrews ZB. *The role of corticostriatal-hypothalamic neural circuits in feeding behaviour: implications for obesity.* **Journal of Neurochemistry**. 2018 Apr 28. doi: 10.1111/jnc.14455

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Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original (submitted publications. The core theme of the thesis is control of feeding an The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Physiology, Monash University under the supervision of Zane Andrews.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research

In the case of Chapter One my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
2	**Agrp neurons enable adaptive responses to acute stress to promote food seeking**	Submitted to Molecular Psychiatry; under review	70%. Experimental data collection, and drafting manuscript	Alex Reichenbach, assisted with data collection; 2% Sarah H Lockie, assisted with experimental design and data collection 1%; Mathieu Mequinion assisted with data collection 2%; Harry Dempsey, assisted with analysis 1%; Sasha Rawlinson, Felicia Reed assisted with data collection and analysis 1%; Luba Sominsky, Alice E McGovern assisted with data collection 0.5%; Sarah J Spencer contributed to manusctipt 1%; Stuart B Mazzone provided HSV viral vector 0.5%, Chris Dayas, Robyn Brown and	Yes: Harry Dempsey, Sasha Rawlinson and Felicia Reed are Monash students

				Romana Stark contributed to manuscript 1%; Zane B. Andrews, experimental design and writing manuscript 20%	

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Rachel Clarke

Student signature: **Date:** 17th April 2020

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Zane Andrews

Main Supervisor signature: **Date:** 17th April 2020

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Abbreviations

AAV = Adeno-associated virus
ACC = Anterior cingulate cortex
Aictx = Anterior insular cortex
Agrp = Agouti-related peptide
ARC = Arcuate nucleus of the hypothalamus
BNST = Bed nucleus of the stria terminalis
cc = corpus callosum
Cg1 = Cingulate gyrus 1
Cg2 = Cingulate gyrus 1
Cre = Cre recombinase
CRH = Corticotrophin releasing hormone
CORT = Corticosterone
CTb = Cholera-toxin B
DMH = dorsal medial hypothalamus
Fmi = forceps minor
GABA = Gamma-aminobutyric acid
GFP = Green fluorescent protien
HFD = High fat/high sugar diet
HPA = Hypothalmic-pituitary-adrenal
HSV = Herpes simplex virsus
LepRb = Leptin receptor B
LH = Lateral Hypothalamus
MCH = Melanin concentrating hormone
MeA = Medial amgydala
mPFC = medial Pre-frontal cortex
NAc = Nucleus accumbens
NPY = Neuropeptide Y
Nts = Neurotensin
POMC = Proopiomelanocortin
PVN = Paraventricular nucleus of the hypothalamus
SST = Somatostatin
Vgat = Vesicular GABA transporter
Vglut1 = Vesicular glutamate transporter 1
Vglut2 = Vesicular glutamate transporter 2
WT = Wild type

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1. Chapter One: Literature Review

1.0 Introduction

The current estimation of the global obesity epidemic is that 1.9 billion adults are overweight, with 650 million of these classified as obese (*World Health Organisation*, 2000). Excess bodyweight now kills more people than underweight (*World Health Organisation*, 2000) and is an underlying factor contributing to cardiovascular disease, diabetes and many types of cancers. Current recommendations to reduce overweight and obesity focus on lifestyle measures such as diet and exercise. However, framing obesity as a lifestyle disease puts the onus on the individual to change their behaviour and does nothing to acknowledge the genetic and environmental determinants that contribute to obesity. To combat the obesity epidemic we need to improve our understanding of the neural circuits that allow for the overconsumption of food beyond physiological requirements.

What we eat is governed by both metabolic need and hedonic motivation. While much is known about the metabolic control of food intake in terms of the hormones and neurotransmitters that are involved in hunger and satiety, the neural mechanisms underlying the decision to eat for pleasure, often in the absence of hunger remains unclear. Animals and humans innately find high energy foods rewarding, ensuring they are motivated to seek and consume these foods, a valuable adaptation in pre-modern times. However, highly palatable and energy dense foods are prevalent in current environments, meaning this previously adaptive trait has become a liability to many modern humans. It is clear that the overconsumption of high fat, high sugar foods is contributing to the obesity epidemic (Swinburn et al., 2009). Yet current strategies to target obesity focus only on correcting energy imbalance through diet and exercise and do nothing to address the cravings associated with unhealthy foods. A common feature among overweight or obese individuals is that they will continue to eat past their energy requirements, despite understanding the adverse health and social consequences. Many overweight or obese individuals report that while they desire to restrict their food intake in order to lose weight, they frequently fail at their attempts to diet (Puhl et al., 2008). Compulsive food intake that is undeterred by known negative consequences resembles behaviours associated with compulsive drug taking (O'Connor & Kenny, 2016). The inability to control food cravings and make appropriate food choices may be underlying the accumulation and maintenance of excessive body weight. It is possible that the neural circuits involved in processing food reward and decision-making become dysfunctional and contribute to ongoing body weight gain. Therefore, understanding the neural circuits that are involved in food cravings and food choice is crucial to advancing our knowledge of obesity and developing effective treatments.

Lessons from Human Imaging Studies

Functional magnetic resonance imaging (fMRI) studies, which measure brain blood flow as a surrogate of activity, have provided some insight into brain regions that may be involved in food choice and food cravings. These studies infer brain activity by measuring changes in blood-oxygen-level-dependent (BOLD) signals in

response to tasks or stimuli. Another application of fMRI is resting-state functional connectivity MRI (fcMRI) studies, which match patterns of the fluctuations in BOLD signals between brain regions to identify putative brain networks while the subject is at rest. Human fMRI studies have demonstrated that food and food-related cues (visual or olfactory) activate both the reward-associated cortical brain regions and the hypothalamus, the key area involved in energy homeostasis (Huerta et al., 2014; Kenny, 2011; Schur et al., 2009). In addition, recent resting-state fcMRI studies have identified distinct neural networks between the hypothalamus and cortical regions (Harding et al., 2018; Kullmann et al., 2014). Importantly, fasting enhances activity in cortical regions in response to food images (Goldstone et al., 2009; LaBar et al., 2001), whereas satiety and overfeeding reduce food-cue induced activity in both the cortical and hypothalamic regions (Cornier et al., 2009; LaBar et al., 2001; Li et al., 2012; Page et al., 2011). Taken together this demonstrates that the reward value of food is influenced by metabolic state and suggests that food choices may be influenced by neural communication between the hypothalamus and cortical regions.

In obesity, pictures of highly palatable foods increase BOLD activity in corticostriatal regions, which encompass the pre-frontal cortex (PFC), anterior cingulate cortex (ACC), and insular cortex, compared to normal weight controls (Hare et al., 2009; Rothemund et al., 2007; Stoeckel et al., 2008; Yokum et al., 2011). While these regions encode the reward value of a range of stimuli and are involved in decision-making processes (Kenny, 2011; Sescousse et al., 2010), they each appear to have distinct roles in food reward. Within the PFC the medial prefrontal cortex (mPFC) appears to be particularly important in the value attributed to palatable food (Hare et al., 2009). While activity of the dorsolateral prefrontal cortex (dlPFC) increases in food-related tasks where self-control is exercised, suggesting it is a brain region important in dietary restraint (Hare et al., 2009). The ACC BOLD response to pictures of food is overwhelmingly reported to be increased in obese individuals compared to healthy controls (Brooks et al., 2013; Martin et al., 2010; Rothemund et al., 2007; Stoeckel et al., 2008). The ACC is thought to encode food craving as there is higher activity in this region in food addicted individuals compared to non-food addicted individuals when looking at images of food (De Ridder et al., 2016). Finally, the insular cortex, is responsive to taste as well as images of food and is sensitive to current metabolic status and post-ingestive feedback (Avery et al., 2017; Frank et al., 2013; Simmons et al., 2013). The fact that obesity is associated with increased activity in these regions in response to food-cues suggests that obese individuals may be highly sensitive to the rewarding properties of palatable foods. More importantly, obese individuals also have altered functional connectivity between the hypothalamus and cortical regions, suggesting that dysfunction in these circuits could be contributing to obesity (Kullmann et al., 2014). However, there are several limitations in the interpretation of fMRI and fcMRI data. Firstly, BOLD signals do not indicate the types of neurons that might be active or the neurotransmitters or neuropeptides involved in a response. Secondly, head movements and physiological artefacts of cardiac rhythm and respiration are known confounders of fcMRI studies (Buckner et al., 2013). Other factors such as the task given to subjects during measurements and recent experiences can modulate fcMRI results (Buckner et al., 2013). Also, the human hypothalamus is relatively small and can be very difficult to image, limiting what can be concluded about the overall activity of this region (Huerta et al., 2014). Finally, functional connectivity does not directly represent anatomical connectivity as a number of regions that are not connected still

observe functional correlations (Buckner et al., 2013). While human imaging studies are useful in qualitatively describing the differences in neural activity patterns of obese patients, they provide no mechanistic insight into the underlying circuitry implicated in the disease. Thus, animal models are necessary to probe the functional relevance of neural circuits linking energy homeostasis, decision-making and reward to overconsumption of palatable food.

Identification of the Lateral Hypothalamus as a Feeding and Reward Centre

Animal studies have been crucial to advancing our understanding of the neurocircuitry involved in the hypothalamic regulation of food intake. Rodents and humans have many fundamental similarities in the organisation of cortical and feeding circuits, as well as in their performance of decision-making, and thus rodent models are useful to investigate the function of neural circuits (Balleine & O'doherty, 2010; Carandini & Churchland, 2013). Classical electrical stimulation and lesioning studies identified the lateral hypothalamus (LH) as a key region involved in feeding and reward (Anand & Brobeck, 1951; Hoebel & Teitelbaum, 1962; Margules & Olds, 1962; Olds & Milner, 1954). The LH is a heterogeneous region, encompassing a number of distinct neuronal populations as well as fibres of passage, including the medial forebrain bundle and fornix (Berthoud & Münzberg, 2011). As one of the most extensively connected areas within the hypothalamus the LH is uniquely positioned to receive information related to both internal metabolic status and external environmental conditions. A major function of the LH is thought to be the integration of interoceptive and exteroceptive information and the subsequent coordination of an appropriate behavioural response (Berthoud & Münzberg, 2011). More recently, the development of techniques that can remotely manipulate cell populations such as optogenetics and chemogenetics have significantly advanced our understanding of the function of the LH. Numerous recent studies demonstrate that the activation or inhibition of distinct cell types within or projecting to the LH significantly modifies feeding behaviour and motivation to seek reward (Carus-Cadavieco et al., 2017; Jennings et al., 2013, 2015; Nieh et al., 2015; O'Connor et al., 2015; Stamatakis et al., 2016; Wu et al., 2020; Wu et al., 2015). Specifically, inhibitory inputs to LH neurons can drive self-stimulation and feeding in well-fed mice (Jennings et al., 2013), suggesting the neural circuits involving the LH play an important role in non-homeostatic feeding and reward. In support of this, human imaging studies have found functional connections between the LH and cortical regions, particularly mPFC and ACC, which are altered in obesity (Harding et al., 2018; Kullmann et al., 2014). This literature review will argue that the LH is a crucial site in the integration of hedonic motivation and metabolic feedback, and will investigate the possibility that perturbations in connectivity between the LH and cortical regions are involved obesity. In addition, there will be a brief discussion of the homeostatic control of food intake, and how neurons in these circuits influence behaviour.

1.1 Classical Studies of the Lateral Hypothalamus

Early studies in rodents identified the LH as a "feeding centre"(Anand & Brobeck, 1951). Bilateral electrolytic lesions to the LH suppressed feeding and caused weight loss (Anand & Brobeck, 1951), whilst electric stimulation rapidly induced feeding well beyond metabolic requirements (Delgado & Anand, 1952). Shortly

after these reports, evidence emerged from self-stimulation studies suggesting the lateral regions of the hypothalamus were also associated with reinforcement or reward (Margules & Olds, 1962). The electrical stimulation of the LH could induce both voracious feeding and self-stimulation in rats; an observation that was augmented by fasting and inhibited by excessive feeding (Hoebel & Teitelbaum, 1962; Margules & Olds, 1962), suggesting shared common neural circuitry.

Although electrical stimulation and lesion studies have been useful in identifying the LH as a key region involved in feeding and reward, the techniques have limitations as to the questions that can be answered (Berthoud & Münzberg, 2011; Stuber & Wise, 2016). Firstly, both the medial forebrain bundle, which projects from the brainstem to the olfactory bulb, and the fornix fibres that connect the hippocampus to the mammillary bodies, pass through the LH (Berthoud & Münzberg, 2011; Nieuwenhuys et al., 1982). In fact, some of the functional characteristics attributed to the LH by these early lesion experiments were later found to partially be a result of the involvement of these fibres of passage (Ungerstedt, 1970). However, chemical lesions to the LH that specifically target cell bodies without damaging passing fibres also resulted in a suppression of feeding (Grossman et al., 1978; Grossman & Grossman, 1982; Stricker et al., 1978; Stuber & Wise, 2016), suggesting that both the neurons within the LH and the fibre bundles, which pass through are involved in regulation of feeding behaviour (Stuber & Wise, 2016). Secondly, electrical stimulation indiscriminately activates the fibres surrounding the electrode tip and gives very little information as to the types of fibres activated (Ranck Jr, 1975). This meant that the substrates and circuitry of the LH involved in feeding and reward remained largely unknown until major advances in the techniques and tools available to neuroscientists.

1.2 Recent Advances in Genetic Techniques for Neuroscience

Our understanding of the neurotransmitters and neuropeptides expressed by neurons in the LH and their function has improved significantly following the advancements in molecular biology and genetics. The development of site-specific genetic recombinase techniques, such as the cre-lox recombination system, has allowed for the manipulation of genes of interest. Cre recombinase is a bacterial enzyme that catalyses the recombination of DNA at a specific site called *lox* (Sauer & Henderson, 1988). The *lox* site consists of two *loxP* sequences, the orientation of which can be altered to manipulate DNA in the desired fashion. For example, if two *loxP* sequences on the same chromosome are repeated the intervening DNA segment will be excised following recombination, while if the two *loxP* sequences are reversed the intervening DNA segment will be inverted and incorporated into the cell (Sauer & Henderson, 1988). The cre transgene can be expressed exclusively in specific neuronal cell types using a cell-specific transcriptional promoter therefore allowing for cell-specific genetic knockouts, or the introduction of a DNA sequence, such as a reporter protein into a certain population of cells (Tsien et al., 1996). The development of the cre-lox system was of significant value to neuroscience due to the number of distinct types of neurons and neuronal circuits present in the brain. Recognising the potential for this technique to revolutionise neuroscience the National Institute of Health (NIH) initiated the Cre-driver Project in 2004, and has now successfully generated a collection of genetically modified mice expressing the cre-transgene in specific neuronal cell-types (Tsien, 2016). Cre-

driver mice can be used for strategic breeding, for example to generate knockout or reporter mice. However, they can also be used in combination with cre-dependent viral vectors delivered via stereotaxic injection to enable conditional deletion or expression of genes in specific regions of the brain. To date, the cre-lox system has contributed significantly to our understanding of distribution, connectivity and function of many neuronal cell types.

Several powerful tools have emerged over the last decade utilising the cre-lox system. Optogenetics uses light-gated ion channels called opsins, originally found in microbial organisms, to acutely control the activity of neurons (Deisseroth, 2011), which can be utilised to examine the function of both specific populations of neurons and specific neural circuits *in vivo*. For example, light-sensitive opsins (either excitatory or inhibitory) can be inserted into cre-expressing neurons of a mouse using a cre-dependent viral vector (Deisseroth, 2011). Specific cell bodies can then be activated or inhibited by delivering light via optical fibres placed directly above the neurons of interest (53). Alternatively, by placing optical fibres above known projection site of the light-sensitive neurons it is possible to selectively activate or inhibit the axon terminals of a subset of the genetically modified neurons, therefore isolating a specific neural circuit (Deisseroth, 2011). Chemogenetic techniques, again in combination with the cre-lox system, have also emerged as useful tools in activating or inhibiting specific neurons. For example, Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are genetically engineered G-protein coupled receptors that can be inserted into a specific population of neurons and then activated with clozapine-N-oxide (CNO), an otherwise physiologically inert compound (Urban & Roth, 2015). This allows for non-invasive chronic activation or inhibition of neurons *in vivo*. Other key advancements include cre-dependent retrograde and anterograde viral tracing techniques that make it possible to visualise the circuitry of specific cell types (Sun et al., 2014), and the development of genetically encoded calcium indicators (GCamp), which allows the activity of specific neurons to be measured *in vivo* (Tian et al., 2009). Together these tools have given us a more detailed understanding of the neurons of the LH and their input and output circuitry, and will continue to aid in future investigation of complex neural circuitry.

1.3 Anatomy of the Lateral Hypothalamus

The LH is a relatively large, heterogeneous structure that lies anterior to the ventral tegmental area (VTA) and posterior to the pre-optic area. Compared to the other sub-regions within the hypothalamus, which have densely packed populations of genetically distinct cells, the LH is not as anatomically well-defined (Stuber & Wise, 2016). Within the LH there exists numerous discrete neuronal populations that are diffusely scattered throughout the region (Bernardis & Bellinger, 1993; Berthoud & Münzberg, 2011)). These can be described by their neurotransmitter or neuropeptide expression.

Glutamatergic and GABAergic Neurons

Neuronal markers for glutamatergic (Vesicular glutamate transporter type 2 or *Vglut2*) and GABAergic (Vesicular GABA transporter or *Vgat*) cells are abundantly expressed in the LH (Stuber & Wise, 2016). These

inhibitory and excitatory cells are intermingled throughout the LH and appear to have opposing effects on behaviour (Stuber & Wise, 2016). The LH contains the highest concentration of GABA neurons within the hypothalamus (Kimura & Kuriyama, 1975), and evidence overwhelmingly demonstrates these neurons promote feeding. Acute activation of LH^{Vgat} neurons via optogenetics induces feeding behaviour in fed mice, time spent in a location paired with optogenetic activation and self-stimulation (Jennings et al., 2015), much like the effect of crude electrical stimulation (Delgado & Anand, 1952; Hoebel & Teitelbaum, 1962; Margules & Olds, 1962). While optogenetic inhibition of the LH^{Vgat} neurons had the opposite effect, reducing feeding and the time spent in a location paired with photoinhibtion; and cell-specicifc ablation of LH^{Vgat} neurons reduces food intake, body weight and motivation to obtain rewards (Jennings et al., 2015). Interestingly, long-term activation of LH^{Vgat} neurons via chemogentics results in increased consummatory behaviours (licking of a palatable liquid), but not the motivation to obtain a reward (Jennings et al., 2015). From *in vivo* calcium imaging experiments, there appears to be distinct subpopulations within the LH^{Vgat} expressing neurons that encode for either appetitive (reward-seeking) behaviours or consummatory behaviours (Jennings et al., 2015), however more work is required to define these subsets further. These recent studies suggest a key role for LH *Vgat* expressing neurons as promoters of reward-seeking and feeding behaviour (Jennings et al., 2015).

In contrast, optogenetic activation of the excitatory LH *Vglut2* neurons suppresses feeding in fasted mice and is aversive (Jennings et al., 2013). Whereas inhibition of LH *Vglut2* neurons is sufficient to drive feeding in fed mice and, in addition, increases the preference for palatable food rewards (Jennings et al., 2013). Further supporting the observations of these optogenetic studies, cell-specific ablation of LH Vglut2 increases consumption of energy dense palatable food and results in weight gain while (Stamatakis et al., 2016). Taken together, it appears LH GABAergic neurons drive feeding, while LH glutamatergic neurons suppress feeding. It is possible that this balance between activity of LH GABAergic and glutamatergic neurons ultimately controls feeding and motivated behaviour in response to environmental stimuli (Stuber & Wise, 2016). Along with excitatory and inhibitory neurotransmitter expression, LH neurons can be classified according to neuropeptide content and these also contribute to feeding and reward-seeking behaviours.

Orexin

Localised to the LH and nearby surround regions, orexin (also known as hypocretin) producing neurons are primarily associated with arousal but also function to modify energy homeostasis and motivated behaviours (Sakurai, 2014). Orexin neurons produce the isoforms orexin-A and orexin-B from the same precursor peptide and many are also reported to co-express glutamatergic markers (Rosin et al., 2003). Initial studies classified orexins as orexigenic peptides; central administration of orexins was observed to increase feeding (Sakurai et al., 1998), while centrally delivered orexin receptor antagonists, or genetic ablation of orexin cells reduced food intake (Hara et al., 2001; Haynes et al., 2000). However more recent studies indicate that the actions of the orexins are far more complicated than originally considered. Orexin signalling increases both food intake and energy expenditure, and an overall increase in orexin tone is protective against diet induced obesity (Funato et al., 2009). Moreover, long-term lack of orexin signalling results in the development of obesity (Hara

et al., 2001). Orexins are also implicated in rewarding aspects of feeding. Central delivery of orexin-A increases the motivation of rats to work for a highly palatable food-reward (Choi et al., 2010), whilst inhibiting orexin signalling reduces reward-seeking behaviour (Borgland et al., 2009). Orexin neurons are activated by the expectation of palatable foods and other rewards (Choi et al., 2010; Harris et al., 2005), and are therefore likely involved in supporting motivated behaviour required to obtain food, rather than influencing the effect of receiving the reward itself (Sakurai, 2014). As orexin neurons appear to be responsive to internal signals of energy balance, such as glucose, leptin and ghrelin (Yamanaka et al., 1999), as well as the act of eating (Gao & Horvath, 2016), they may be involved in mediating metabolic and hedonic signals to orchestrate the appropriate behavioural response. However, the primary function of the orexin neurons is to regulate arousal state. Optogentic activation of orexin neurons is sufficient to drive wakening from sleep (Adamantidis et al., 2007) and the effect of the orexins on food intake is influenced by the time of day (Yamanaka et al., 1999). Therefore, it is likely that the contribution of the orexins to LH-mediated feeding and reward-seeking behaviour are secondary to the regulation of arousal (Berthoud & Münzberg, 2011). In summary, the orexin system contributes to feeding and reward-seeking behaviour, however it's primary function is likely regulation of arousal.

Melanin Concentrating Hormone

Like orexin, melanin concentrating hormone (MCH) producing neurons are almost exclusively found in LH and regulate arousal as well as feeding behaviour. In contrast to the orexins, optogenetic activation of MCH neurons promotes sleep (Jego et al., 2013). MCH burst firing also occurs in awake animals, as determined using GCamp signalling *in vivo*, and is inversely associated with orexin activity in response to different stimuli (González et al., 2016). MCH behaves more like a typical orexigenic peptide; central administration increases feeding (Qu et al., 1996) and bodyweight on both chow and high fat diet (Gomori et al., 2003), while MCH knockout mice eat less than wild types and are lean (Shimada et al., 1998). It seems unlikely that MCH plays a role in purely hedonic, pleasurable associated aspects of food intake (Brown et al., 2015). For example, while the central delivery of orexin administration reportedly drives a preference for high-fat food compared to chow in mice, MCH treatment resulted in an overall increase in food intake from both diets (Clegg et al., 2002). Interestingly, when MCH-treated rats were given a choice between sucrose, glucose and saccharin there was a significant preference for the energy containing solutions rather than the sweet taste, suggesting that MCH may bias consumption toward energy content (Sakamaki et al., 2005). Supporting this, a recent study demonstrated that MCH neurons are necessary for sensing the nutritional value of sugar (Sakamaki et al., 2005). It is important to note that both the sensory information relating to sweet taste and the post-ingestive signals relating to nutrient content increase striatal dopamine release through separate circuitries (Tellez et al., 2016). For example, when given a choice between sucrose, and an artificial sweetener such as sucralose, mice prefer to consume sucrose due to the greater caloric (reward) feedback. Ablation of MCH neurons results in mice that cannot detect the reward value (i.e. no increase in striatal dopamine levels) of an energy containing sucrose solution (Domingos et al., 2013). While, activation of MCH neurons when paired to consumption of an energy-free sucralose solution results in increased total striatal dopamine release compared to consumption of sucralose alone or activation of MCH neurons alone (Domingos et al., 2013).

Therefore, both the activation of MCH neurons and sweet taste are required to drive reward (Domingos et al., 2013). The mechanisms behind this are not yet known, but could be in part due to the ability of MCH neurons to sense glucose levels (Domingos et al., 2013). Consistent with this MCH mRNA is increased with fasting suggesting its main role in feeding is to maintain energy homeostasis (Qu et al., 1996). There appear be both excitatory and inhibitory populations of MCH neurons, as some MCH neurons co-express the GABAergic marker glutamate decarboxylase-67 (GAD-67- an enzyme that catalyses the decarboxylation of glutamate to GABA) (Jego et al., 2013), while others have been found to express glutamatergic markers *Vglut1* (Harthoorn et al., 2005) and *Vglut2* (Collin et al., 2003). It has been suggested that MCH regulation of sleep occurs primarily through the release of the neurotransmitter GABA (Brown et al., 2015; Jego et al., 2013), though whether MCH neurons co-release GABA is contentious. Neither MCH or orexin neurons express the vesicular GABA transporter *Vgat*, and so the acute feeding response seen with optogenetic activation of LH *Vgat* neurons is likely occurring independently of these neuropeptides (Stuber & Wise, 2016). While GAD-67 mediates GABA production within a cell, it does not cause GABA release, therefore if MCH cells secrete GABA this must occur through another GABA transporter enzyme, not *Vgat,* but this requires further investigation. Like orexin, it seems the feeding and metabolic phenotypes associated with MCH are secondary to its influence on arousal state.

Neurotensin

Neurotensin (*Nts)*, another neuropeptide produced in the LH, is implicated in feeding and body weight regulation (Brown et al., 2015; Schroeder & Leinninger, 2018). *Nts* neurons, which are distinct from the MCH and orexin producing cells, are not restricted to the LH but also found in the midbrain and limbic system (Brown et al., 2015; Schroeder & Leinninger, 2018). *Nts* is considered an anorexigenic neuropeptide; central delivery of *Nts* reduces food intake in fasted and fed rats (Cooke et al., 2009), and disruption of *Nts* signalling through knock-out of neurotensin receptor-1 (*Ntsr1*) results in increased food intake and weight gain (Kim et al., 2008). *Nts* works with leptin, a signal of positive energy balance, to modify feeding. In fact, the majority of LH neurons that contain leptin receptors (*LepRb*) also express *Nts*, and this subset of neurotensin neurons appear to be restricted the LH (Leinninger et al., 2011). Mice lacking *LepRb* only in *Nts* neurons have slightly elevated food intake, increased fat mass and reduced activity compared to controls (Leinninger et al., 2011), highlighting the importance of LH^{Nts} neurons in mediating leptin action. Central *Nts* signalling through *Ntsr1* is implicated in non-homeostatic feeding (Opland et al., 2013). *Ntsr1* knock-out mice have increased sensitivity to rewarding properties of highly palatable food and sucrose compared to wild types, suggesting that *Nts* signalling is important in attenuating the over-consumption of palatable energy dense foods (Opland et al., 2013). Again, this is likely leptin-mediated, as *Ntsr1* potentiates leptin-induced suppression of food intake (Kim et al., 2008). It appears that leptin/*Nts* effects on feeding behaviour are exerted via projections from *LepRb*-positive *Nts* neurons to VTA dopamine neurons, which are important in motivated behaviour (Leinninger et al., 2011). Moreover, optical activation of the LH to VTA pathway promotes self-stimulation, an effect that is attenuated by blocking *Nts* signalling in the VTA (Kempadoo et al., 2013). This observation seems to conflict with the observation that *Nts* signalling suppresses consumption of natural rewards. However, activation of the dopamine system cannot predict the promotion or suppression of feeding (Date et

al., 1999; Horvath et al., 1999). Like *Nts*, orexin activates VTA dopamine neurons and animals will self-administer orexin into the VTA (Borgland et al., 2009). Conversely, orexin promotes the intake of rewarding substances such as palatable food and drugs of abuse (Borgland et al., 2009; Harris et al., 2005) while central *Nts* signalling suppresses overconsumption of palatable food (Opland et al., 2013). Therefore, *Nts* modifies intake of palatable foods although it remains unclear as to precisely how it achieves this. A recent study using chemogenetics to acutely activate the LH^{Nts} neurons reports a suppression of chow intake, suppression of motivation to obtain sucrose reward, body weight loss and increased locomotor activity (Woodworth, Beekly, et al., 2017). However, in this study activation of LH^{Nts} neurons did not affect consumption of a palatable diet, suggesting that *Nts* signalling in other brain regions may mediate suppression of hedonic feeding (Woodworth, Beekly, et al., 2017).

Galanin

Galanin (*Gal*) expressing neurons are widely distributed throughout the brain and within the LH they are highly co-localised with *Nts* neurons (approximately 95%) (Laque et al., 2013; Stuber & Wise, 2016). Like neurotensin, galanin modulates the mesolimbic dopamine system (Stuber & Wise, 2016). This is likely due to galanin projections from the PVN to VTA (Berthoud & Münzberg, 2011) as the LH^{Gal} neurons do not project directly to the VTA, but rather onto orexin neurons where their inhibitory actions are thought to mediate motivated food seeking and body weight (Laque et al., 2015). There is conflicting data on whether *Gal* is an orexigenic or anorexigenic neuropeptide, and this may be dependent on the site of *Gal* action or the sub population of *Gal* neurons investigated. Infusion of *Gal* into the PVN induces feeding, while infusion into the LH has no effect (Kyrkouli et al., 1990). Due to co-localisation with *Nts*/*LepRb* neurons and the observation that LH^{Gal} neurons project to orexin neurons, some studies suggest that LH^{Gal} neurons mediate the anorexigenic actions of leptin via *Gal* mediated inhibition of orexin neurons (Laque et al., 2013, 2015). A more recent study used chemogenetics to activate LH^{Gal} neurons and observed an increase in motivated food seeking and locomotor activity but not in total food consumption (Qualls-Creekmore et al., 2017). Further investigation of the LH^{Gal} neurons based on projection targets is warranted to clear up the role for LH^{Gal} neurons in appetitive and consumatory feeding behaviours.

To summarise, the LH contains several neuronal cell-types that are responsive to signals of energy homeostasis and involved in coordinating feeding and motivated behaviours. While understanding the functions of the various LH cell-types has given insight into LH control of feeding and reward, the different neuronal populations do not act alone and are densely connected both locally within the LH and to regions beyond the LH. To fully comprehend the physiological function of neurons of the LH it is important to consider the input and output circuitry of these cells.

1.4 Connectivity of the Lateral Hypothalamus

The LH receives substantial excitatory and inhibitory inputs from both hypothalamic and extra-hypothalamic regions providing information about metabolic state and environmental conditions (Figure 1.1). The LH then integrates this information to coordinate appropriate behavioural output via its direct projections to regions

associated with motivated behaviours. Originally the circuitry into and out of the LH was described using tracing techniques. Our understanding of the function of LH circuits has been revolutionised by the development of techniques such as the cre-lox system, optogenetics, and chemogenetics.

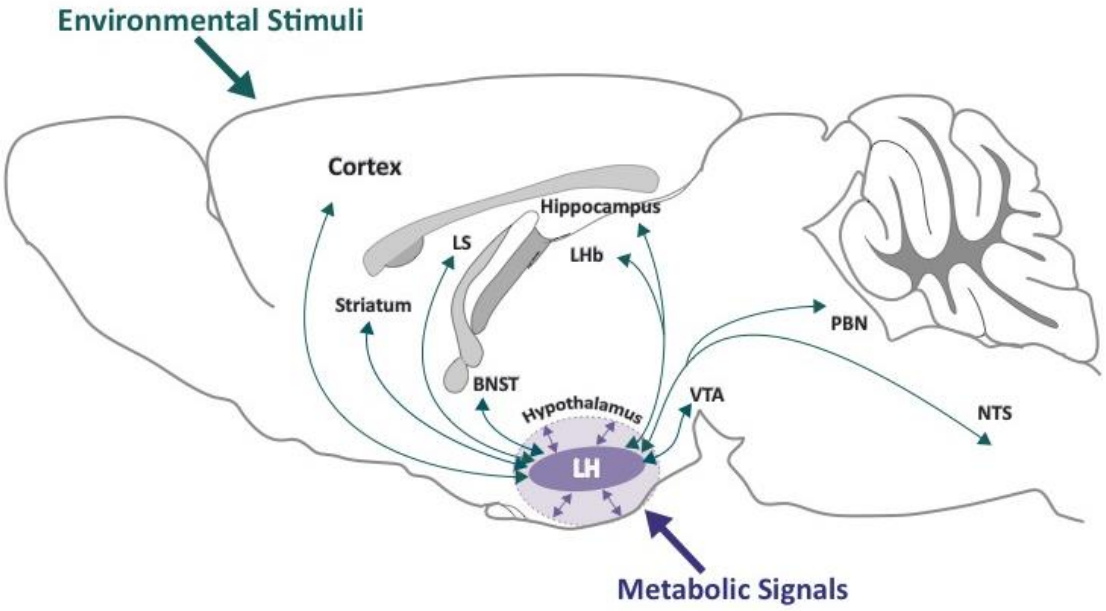

Figure 1.1 Extra-hypothalamic and hypothamic connectivity of the lateral hypothalamus (LH). The LH receives information from cortical and subcortical regions pertaining to environmental cues. The LH also receives signals of metabolic state from other hypothalamic sites. The LH projects to a broad range of brain areas involved in motivation, feeding, emotion, stress and memory. Abbreviations: Bed Nucleus of the Stria Terminalis (BNST), Lateral Habenula (LHb), Lateral Septum (LS), Parabrachial Nucleus (PBN), Nucleus of the Solitary Tract (NTS), Ventral Tegmental Area (VTA). Figure produced by Rachel Clarke and published in Clarke et al., 2018. Adapted from Allen Brain Atlas.

Extra-hypothalamic inputs

Early retrograde tracing studies in animals show that there are several cortical and subcortical regions that project to the LH including the mPFC (comprised of the prelimbic and infralimbic cortices), orbital frontal cortex (OFC), ACC (comprised of the cingulate gyrus 1,2 & 3), insular cortex, nucleus accumbens (NAc) of the ventral striatum, lateral and dorsal septal nuclei, extended amygdala, bed nucleus of the stria terminalis (BNST), hippocampus and lateral habenula (Barone et al., 1981; Gabbott et al., 2005; Kita & Oomura, 1982). Many of these regions, along with the insular cortex, and ACC, have since been reported to project to orexin neurons within the LH (Yoshida et al., 2006). The development of transgenic mice has allowed for a more detailed map to be developed in terms of the neurotransmitters and neuropeptides involved in LH circuitry. Sakuri et al (2005) generated mice expressing a transgene containing a fusion protein between tetanus toxin C (TTC) and green fluorescent protein (GFP) exclusively in orexin neurons (Sakurai et al., 2005). This allows for the selective transfer of the fusion protein to cells that project to orexin neurons, therefore labelling these cells with GFP (Maskos et al., 2002). Labelling was observed in the cholinergic neurons of the basal forebrain, GABAergic neurons of the preoptic area, serotonergic neurons of the raphe nuclei and many neurons within subcortical areas involved in emotion, reward and motivation, such as the amygdala, lateral septum, BNST and nucleus accumbens (Sakurai et al., 2005). The diversity of afferent projections demonstrates the wide range of substrates and neurotransmitters that must regulate the activity of the orexin peptides, and other neurons within the LH. Many of these inputs were confirmed using injections of conventional retrograde and anterograde tracers in rats (Yoshida et al., 2006). However some of the projection sites reported in the study

by Sakuri et al (2005), such as the medial septum, do not contain known orexin afferents and could represent ectopic expression of the transgene or transfer to second order neurons(Sakurai et al., 2005; Yoshida et al., 2006). A more recent study using robust cre-dependent retrograde tracers in orexin-cre and MCH-cre mice has produced a detailed global map of inputs to both neuron types (González et al., 2016), again confirming many of the findings of earlier tracing studies. In addition, the results demonstrate that there are inputs to orexin and MCH neurons from key cortical areas identified to be involved in human obesity, including the anterior cingulate cortex, the insular and the medial pre-frontal cortex (González et al., 2016). Given that there are a large population of neurons in the LH, which do not express orexin or MCH, repeating this approach in *Vgat*-cre or *Vglut2*-cre mice would contribute significantly to our understanding of LH circuitry.

Neural tracing studies have been valuable to the understanding origins of the LH input circuitry. However, it is important to determine whether these anatomical connections are functionally relevant. Advances in neuroscience have allowed for a more detailed picture of the nature of the inputs to the LH and their role in modifying feeding and reward seeking behaviour. Jennings et al. (2013) show projections from the BNST, a region involved in motivation and anxiety, are largely GABAergic and project to glutamatergic LH neurons (Jennings et al., 2013). Activation of this inhibitory circuit using optogentics rapidly induces feeding in well fed mice, and also results in self-stimulation behaviours in a manner similar to optogenetic inhibition of LH glutamate neurons (Jennings et al., 2013). Moreover, activation of BNST GABA projections to the LH markedly increased preference for palatable food in well-fed mice, suggesting that this circuit may be implicated in overriding metabolic signals and driving hedonic eating (Jennings et al., 2013).

The lateral septum (LS) also provides inhibitory input directly to the LH that modfiy feeding (Carus-Cadavieco et al., 2017; Sweeney & Yang, 2016). LS^{Vgat} neurons were found to project to and inhibit a subset of LH^{Vgat} neurons, resulting in the suppression of food intake (Sweeney & Yang, 2016). This circuit does not affect anxiety behaviours or locomotor activity, however whether this circuit may influence motivated food-seeking behaviour was not investigated (Sweeney & Yang, 2016). The somatostatin (*SST)* expressing LS neurons represent a subset of LS^{Vgat} neurons that also project to LH^{Vgat} neurons (Carus-Cadavieco et al., 2017). However, activation of LS^{SST} neurons results only in changes to food-seeking behaviour and not to food intake - demonstrating that different subpopulations of inhibitory LH projection neurons may serve different functions (Carus-Cadavieco et al., 2017). The results of the study looking at LS^{SST} projections to the LH will be discussed in more detail below as there appears to be upstream inputs from the mPFC that may explain these results (Carus-Cadavieco et al., 2017).

The results from the studies discussed here are consisted with the notion that within the LH, GABA signalling drives feeding and motivated behaviour, while LH glutamatergic activity has the opposite effect. In addition, the majority of direct inputs to the LH identified to play a role in feeding and reward seeking to date, seem to target GABAergic or glutamatergic neurons and not orexin or MCH producing cells (Jennings et al., 2013; O'Connor et al., 2015). Inhibitory inputs that synapse onto orexin neurons, such as those from the pre-optic area, appear to be involved in regulating arousal and not feeding (Saito et al., 2013). There are also

substantial inhibitory inputs to MCH neurons that arise in the BNST and the amygdala, however the role of these circuits in feeding and reward is not clear (González et al., 2016).

Striatal inputs to the LH

The nucleus accumbens shell (NAcSh) of the ventral striatum is an important region implicated in hedonic food intake in both humans and rodents (Contreras-Rodriguez et al., 2019; Kelley, 2004). The NAcSh provides another source of inhibitory input to the LH and it appears this circuit can override metabolic signals and rapidly modulate feeding (Heimer et al., 1991; O'Connor et al., 2015). The functional importance of this circuit in feeding behaviour has been known for some time (Maldonado-Irizarry et al., 1995; Stratford & Kelley, 1997, 1999), but has more recently been investigated in detail (O'Connor et al., 2015; Thoeni et al., 2020). Using a retrograde tracer in combination with transgenic reporter mice, the majority of neurons projecting from the NAcSh were found to be the inhibitory dopamine receptor-1 expressing medium spiny neurons (D1R-MSN) (O'Connor et al., 2015). Optogenetic activation of NAcSh D1R-MSN fibres in the LH was sufficient to suppress food intake and rapidly terminate feeding bouts in food-deprived mice; while optogenetic inhibition of NAcSh D1R-MSN cell bodies increases consumption of a palatable liquid, and promotes undisrupted feeding during a distraction test in well-fed mice (O'Connor et al., 2015). These results suggest activity of the NAcSH to LH pathway can prolong or terminate a feeding bout, despite opposing metabolic signals. More recently, a follow up study demonstrated that the NAcSH to LH pathway undergoes synaptic depression following periods of acute fasting, or high fat diet feeding - scenarios where overeating is promoted (Thoeni et al., 2020). Therefore, this circuit may contribute to the overconsumption of unhealthy foods, or binge eating following a fast and is important to consider as factor underlying obesity.

Cortical inputs to the LH

The function of the majority of LH afferents arising from cortical areas have not yet been elucidated. Cognitive processing within the cortex involves gamma oscillations, which can be defined as fast, rhythmic neural activity of 30-90Hz that is mediated mainly by inhibitory interneurons (Buzsáki & Wang, 2012). The projection neurons of the cortex primarily consist of excitatory pyramidal neurons that express calcium/calmodulin-dependent kinase II alpha (CamKII-α), however there are subsets of GABAergic cortical neurons that project long-range to other structures (Tomioka et al., 2015). A recent study by Carus-Cadavieco et al. (2017) identified a top-down pathway connecting the mPFC, LS and LH that uses coordinated gamma oscillations to organise feeding behaviour (Carus-Cadavieco et al., 2017). By examining the activity of LH neurons *in vivo* the authors demonstrated that there are two sub populations of LH^{Vgat} neurons that respond when a mouse is either close to food, or at a distance, both of which are separately activated during gamma oscillations within the LH (Carus-Cadavieco et al., 2017). Optogenetic activation of the LS inhibitory somatostatin projections to the LH at gamma frequencies stimulated food seeking behaviour, but not food intake (Carus-Cadavieco et al., 2017). Upstream, CamKII-α expressing neurons of the mPFC were demonstrated to project monosynaptically to the LS, and mPFC gamma-rhythmic oscillations were also associated with neuronal activity within the LS (Carus-Cadavieco et al., 2017). Optogenetic stimulation of

mPFC CamKII-α projections in the LS at gamma frequencies resulted in increased food seeking, without an increase in food intake, and also improved performance on a food-reward learning task (Carus-Cadavieco et al., 2017). Taken together this mPFC-LS-LH circuit appears to be important in motivated food seeking behaviours. This study also highlights the advantages of recording from neuronal populations in different brain regions to determine how these might ordinarily behave *in vivo*, rather than simply stimulating whole populations of projection neurons as described in the paper investigating the LS^{Vgat} to LH^{Vgat} circuit above (Sweeney & Yang, 2016). It would be interesting to determine whether the monosynaptic projections from cortical neurons to the LH also use synchronised gamma oscillations to exert control over feeding behaviours.

The function of extra-hypothalamic excitatory afferents to the LH have not been as well characterised as inhibitory circuits. Long-range projection neurons of the cortex are primarily pyramidal glutamtergic neruons, many of which project to the LH (Gabbott et al., 2005). One recent study demonstrates that excitatory inputs from the anterior insular cortex, a brain region implicated in obesity, to the LH^{Vglut2} neurons encode aversion and inhibit food intake in fasted animals (Wu et al., 2020), therefore providing an example of a top-down cortical to LH circuit that can override homoeostatic signals. The projections from the mPFC to LH have also recently been investigated, however only in the context of agression, where they were observed to increase abnormal aggressive behaviours (Biro et al., 2018). Whether this same circuit provides top-down control over feeding and reward-seeking behaviour remains unknown but warrants investigation.

Hypothalamic Inputs

The LH receives substantial excitatory and inhibitory inputs from both local interneurons and other hypothalamic regions including the arcuate nucleus (ARC) (Betley et al., 2013), paraventricular nucleus (PVN) and ventral medial hypothalamus (VMH)(González et al., 2016). There is also dense interconnectivity within the LH (González et al., 2016). Optogenetic activation of orexin neurons inhibits the majority of MCH neurons either directly or indirectly via GABA interneurons, and this occurs in a manner that is dependent on orexin receptor signalling (Apergis-Schoute et al., 2015). Though, as MCH and orexin signalling primarily function to regulate arousal it is unclear if these microcircuits are directly relevant to feeding or reward behaviours. Of the inputs from other hypothalamic nuclei, the ARC-LH circuit has been the most thoroughly investigated. The ARC contains neurons that directly sense energy balance and then act either to promote hunger or satiety accordingly. The agouti related peptide/neuropeptide Y (Agrp/NPY) co-expressing neurons of the ARC sense negative energy balance and promote food intake via both the release of the peptides Agrp and NPY and the release of GABA (Aponte et al., 2011; Betley et al., 2013). Optogenetic activation of the Agrp to LH circuit rapidly induces feeding (Betley et al., 2013), suggesting that this is due to the release of fast acting GABA and NPY in the LH. Consistent with this, local injection of NPY into the perifornical area of the LH drives feeding suggesting there are neurons of this region contain NPY receptors (Stanley, Magdalin, et al., 1993). Given that glutamate signalling in the LH suppresses feeding, it is plausible that the Agrp/NPY terminals predominately target and suppress LH glutamatergic neurons though this needs to be confirmed. The proopiomelanocortin (POMC) neurons of the ARC detect energy surplus and suppress appetite through the release of the peptide alpha-melanocyte-stimulating hormone (alpha-MSH) (Aponte et al., 2011). POMC

neurons also project to the lateral hypothalamus, however whether activation of this circuit is sufficient to suppress feeding is not yet clear (Betley et al., 2013). The ARC neurons as a whole project to both orexin and MCH neurons (González et al., 2016). Relevant to this literature review, a study by Kampe *et al.* (2009) used transynaptic retrograde tracing in rats to identify inputs into cortical regions thought to be relevant to feeding, including the insular cortex,, ACC and NAc of the ventral striatum (Kampe et al., 2009). Substantial numbers of first-order neuron projecting to the cortical and reward-associated striatal regions were found in the lateral hypothalamus, many of which express orexin or MCH, while second-order neurons were present within the PVN and ARC (Kampe et al., 2009). Their results highlighted that Agrp and POMC neurons have divergent projection sites, with orexigenic Agrp neurons projecting to the NAc, and anorexigenic POMC neurons primarily projecting towards the insular cortex, and ACC (Kampe et al., 2009). This study gives evidence of multi-synaptic circuits originating from the energy sensing neurons of the ARC, that reach cortical and striatal regions via the LH. This demonstrates the significance of the LH in relaying metabolic signals to regions involved in reward processing and decision-making. The relevance of these observations to the control of homeostatic and/or hedonic feeding is not yet clear.

Extra-hypothalamic outputs

The neurons of the LH have a vast number of anatomical projections to extra-hypothalamic regions (Berthoud & Münzberg, 2011; Saper et al., 1979; Stuber & Wise, 2016). As just discussed, there are orexin and MCH neurons that project from the LH to cortical regions including the insular cortex, and ACC (Kampe et al., 2009) though the functions of these circuits have not yet been defined. LH projections that are known to be functionally relevant to feeding, reward and motivated behaviours include the LH to VTA, LH to lateral habenula, and LH to striatum circuits. Nieh et al. (2015) demonstrated that the LH sends both excitatory and inhibitory projections to the VTA, which target both GABA neurons and dopamine neurons (Nieh et al., 2015). In the same study optogenetic activation of the LH to VTA circuit was sufficient to drive feeding in well fed mice (Nieh et al., 2015). In addition, activation of this circuit promoted compulsive sucrose seeking in the face of a negative consequence (foot shock), suggesting a role for this pathway in addictive-like behaviours associated with compulsive feeding in obesity (Nieh et al., 2015). Using *Vgat*-cre and *Vglut2*-cre mice the specific functions of the glutamatergic and GABAergic pathways to VTA neurons were then investigated (Nieh et al., 2015). Optogenetic activation of LH^{Vgat}-VTA circuit induced feeding behaviour in sated mice, but did not affect reward seeking behaviour; while activating the LH^{Vglut2}-VTA circuit had no effect on either behaviour (Nieh et al., 2015). However, LH^{Vgat}-VTA circuit activation also resulted in maladaptive behaviours such as excessive gnawing, suggesting that both the LH^{Vgat}-VTA and LH^{Vglut2}-VTA circuits ar e necessary for appropriately driven feeding and motivated behaviours (Nieh et al., 2015).

The LH also has glutamatergic projections to the lateral habenula, a brain region involved in behavioural avoidance (Stamatakis et al., 2016). Consistent with the role for LH glutamate neurons to inhibit feeding, optogenetic inhibition of the LH^{Vglut2}projections in the lateral habenula resulted in increased consumption of a highly palatable liquid in fed mice (Stamatakis et al., 2016). This circuit is also involved in reward and motivated behaviours, as optogenetic inhibition resulted in real-time place preference, i.e. mice preferred to

spend time in a location previously paired with light-delivery, while activation of the LH^{Vglut2}-VTA pathway was aversive (Stamatakis et al., 2016). This is likely mediated by neurons within the lateral habenula that project directly to VTA GABA neurons, which in turn can act to inhibit VTA dopamine neurons therefore modulating reward (Stuber & Wise, 2016). LH neurons are also involved in striatal dopamine release, since MCH neurons are required for sucrose-mediated dopamine release within the striatum (Domingos et al., 2013). Though whether this is a result of direct projections from the LH^{MCH} neurons to the striatum remains to be determined, it is likely that MCH neurons project to the VTA and modify dopamine release from neurons that project to the striatum.

So far, the LH outputs to reward-related regions of the brain are well studied and clearly, LH projections to these areas play a role in feeding behaviour and reward processing. Delineating the functions of the LH outputs to cortical regions is now possible given the range of tools becoming available to neuroscience. Recently, a study by Petrovich *et al* (2020) demonstrated that the LH orexin expressing neurons project to the mPFC, and that orexin signalling with in the mPFC is required for cue-driven food consumption (Cole et al., 2020). The findings of this study suggest the LH and mPFC connections may be involved in environmentally driven, non-homeostatic signals to eat and may be a factor contributing to the development and maintenance of obesity.

Hypothalamic outputs

The LH projects to most of the hypothalamus including the ARC, PVN, and dorsomedial and ventromedial nuclei (Berthoud & Münzberg, 2011; Saper et al., 1979; Wang et al., 2015). So far only the functional characteristics of the LH projections to the PVN have been investigated. For example, projections from the LH to the PVN are largely GABAergic, and optogenetic activation of this circuit rapidly induces feeding (Wu et al., 2015). Whether these LH^{GABA} neurons co-express neurotensin or MCH or alternatively, exclusively express GABA remains unknown. Orexin neurons have been reported to project directly to the ARC and may have an important role in feeding, however this remains to be investigated (Date et al., 1999; Horvath et al., 1999).

To summarise, both the extra-hypothalamic and hypothalamic GABAergic inputs into the LH investigated so far are important in regulating feeding and reward. Less is known about the glutamatergic inputs from extra-hypothalamic regions and this certainly deserves future attention. The LH also has numerous outputs, which appear to control feeding behaviour and influence reward processing and motivated behaviours. The LH circuits are sensitive to external cues and signals of energy balance again supporting the idea of the LH being the key integrator of interceptive and exteroceptive information. What is still unknown is the functional relevance of the circuitry connecting the LH to cortical regions involved in decision-making and food choice. Given that these connections appear perturbed in human obesity, mouse models to delineate the functional relevance of the circuits between the LH and the mPFC, ACC, and insular cortex. As discussed in the following section, current evidence in humans and animals supports a role for these regions in processing hedonic aspects of feeding and decision-making.

1.5 Cortical Regions Involved in Food Choice and Reward Processing

Pre-frontal Cortex

A range of functions have been ascribed to the PFC including decision making, valuation of stimuli and inhibitory control (Carlén, 2017; Delgado et al., 2016). Neuroimaging studies in humans have implicated the mPFC and dlPFC in processing of food cues (Harding et al., 2018; Schur et al., 2009). In addition, the PFC appears to be a key brain region that is involved in aspects of human addiction, such as regulation of reward systems and higher-order executive control of behaviour (Goldstein & Volkow, 2011). The mPFC in particular appears to be differentially activated in human obesity. Obese individuals consistently display stronger activation of the mPFC in response to images of food than healthy weight controls (Brooks et al., 2013; Kennedy & Dimitropoulos, 2014; Stoeckel et al., 2008), suggesting they may be more sensitive to food cues. In addition to this, when choosing between unhealthy and healthy food options in a fasted state, obese individuals have stronger activation of the mPFC coupled with weaker activation of the hypothalamus (Harding et al., 2018). Overwhelmingly, human neuroimaging data suggest that obese individuals have greater activity in brain regions associated with reward valuation and weaker activity in regions that regulate energy intake. This pattern of brain activity promotes a state where non-homeostatic hedonic properties of a food play a greater role in driving food intake, rather than internal signals of hunger and satiety. In addition to this obese individuals have been reported to exhibit weaker executive function, which may translate to weaker inhibitory control over food consumption (Lowe et al., 2019). The dlPFC is an important region of the PFC involved in food-related self-control (Hare et al., 2009; Kober et al., 2010). The dlPFC influences self-control by modulating the reward value signal encoded by the vmPFC (Hare et al., 2009). Human imaging studies comparing healthy weight controls to obese individuals report that obese individuals have higher activity of the mPFC and lower activity of the dlPFC when looking at images of food, suggesting obese individuals suggesting higher attention to food, with a weakened control system (Brooks et al., 2013). Supporting this, a study investigating neural activity in individuals undergoing voluntary weight loss, vmPFC and dlPFC functional connectivity predicted successful weight loss outcomes, independent of leptin and ghrelin levels (Neseliler et al., 2019). Taken together, it appears obese individuals are not only more sensitive to food cues, and less sensitive to homeostatice signals, but also exhibit weaker cognitive control.

Whether the rodent can be used to study the role of the prefrontal cortex in behaviour has long been debated (Carlén, 2017; Laubach et al., 2018). Opponents against the use of rodent models argue that cortical structure has undergone phyogenetic development over the course of evolution with the dorsolateral part of the PFC in primates receiving inputs from the mediodorsal nucleus of the thalamus (a historic definition of the PFC) and containing granule cells, while the rodent prefrontal region is entirely agranular and therefore not conserved (Carlén, 2017). However, while the dlPFC region of the primate cortex might represent the evolution of the PFC, rodents could hold the functions of these regions within medial or orbitofrontal cortices (Carlén, 2017). The rodent prefrontal cortex has been demonstrated to be involved in impulse control, rule representation and attention – functions previously ascribed specifically to the dlPFC in primates (Carlén,

2017). While still not clear, the regions of the rodent brain known as prelimbic (PrL) and infralimbic (IL) are widely used to describe the rodent mPFC or PFC at large, however these regions and other more orbital regions may encompass some of the functions attributed to the dlPFC of primates (Carlén, 2017).

There are a small number of animal studies investigating the role of the mPFC in feeding behaviour. Photoactivation of the subpopulation of dopamine receptor 1 (D1) expressing neurons of the mPFC has been demonstrated to increase feeding through projections to the basolateral amygdala (Land et al., 2014). In addition, μ-opioid receptor agonism of mPFC neurons results in feeding and hyperactivity, and these neurons appear to project indirectly to orexin expressing neurons in the LH (Mena et al., 2013). Possibly, there is a bi-directional circuit between the mPFC and LH important to feeding behaviour and learned food-cue responses. As mentioned earlier, orexin signalling in the mPFC is required for cue-mediated feeding responses (Cole et al., 2020). So far, no studies have examined the role of the mPFC to LH circuit in feeding behaviour.

Anterior Cingulate Cortex

Similar to the mPFC, neuroimaging studies in obese and binge eating disorder patients consistently demonstrate differential activity in the ACC in response to food or food cues (Brooks et al., 2013; De Ridder et al., 2016; Dimitropoulos et al., 2012; Harding et al., 2018; Martin et al., 2010; Murdaugh et al., 2012; Stoeckel et al., 2008). These studies overwhelmingly find that obese individuals, compared to healthy weight controls, have increased activity in the ACC when looking at images of food or are presented with foods (Brooks et al., 2013). Additional studies suggest the ACC is involved in food craving and food addiction (De Ridder et al., 2016; Gearhardt et al., 2011). Both resting state and food cue-evoked ACC activity is positively associated with food addiction score (De Ridder et al., 2016; Gearhardt et al., 2011). ACC activity is elevated in a similar manner in alcohol addicted individuals, suggesting the possibility of a common underlying neurophysiological substrate (Gearhardt et al., 2011). In healthy individuals, ACC activity appears to encode the value differences of food options (Harding et al., 2018) and therefore perturbations to activity in this region might cause an individual to place more weight on the rewarding properties of a food, and to ignore the health cost associated, or internal signals of satiety.

The cingulate gyrus 1 and 2 in rodents is most commonly described as the ACC and roughly corresponds to Brodmann area 24 in humans. Only a limited number of animal studies that have investigated a role for the ACC in feeding behaviours. Disruption of excitatory neuronal signalling in the ACC via either chemogenetic activation or inhibition suppresses operant responding for a food reward (Hart et al., 2019). In this same study, calcium imaging identified separate subsets of ACC neurons that are active either prior to operant responding (appetitive) or during sucrose pellet retrieval (consummatory), which may explain why both activation and inhibition of the ACC excitatory neurons disrupted normal behaviour (Hart et al., 2019). In addition, this suggests that specific subsets of ACC neurons may be recruited in either appetitive or consummatory behaviour (Hart et al., 2019). Supporting this idea, parvalbumin (PV) expressing interneurons of the ACC are active immediately following reward consumption in a foraging task; while a subset of *SST*

expressing interneurons increase activity during appetitive reward approach (Kvitsiani et al., 2013). Interestingly, the *SST* neurons of the LS that project to the LH are also involved in food approach behaviours (Carus-Cadavieco et al., 2017), suggesting a common role for *SST* neurons in food seeking, though this will require further investigation. The ACC also appears to be required for normal food foraging and decision-making behaviour in a social setting, as pharmacological lesions in this region results in an increase in amount of highly palatable, rewarding food foraged and a simultaneous decrease in normal social foraging behaviour (Zhong et al., 2017). Together, the limited number of animal studies presented here suggest a role for the ACC in appropriate valuation of a food reward. Whether the ACC-LH circuit might influence behavioural response to foods of differing values remains unknown.

Insular Cortex

The insular cortex is responsive to interoceptive cues as well as environmental stimuli pertaining to reward or motivation, and is often jointly activated with the ACC (Craig & Craig, 2009). The strong association of the insular cortex, with changes in homeostatic signals suggests that connections between the insular cortex, and the hypothalamus are important and likely are involved in food choice and the decision to eat. Recent studies in animals suggest the insular cortex is a key brain region involved in predicting and responding to changes in nutrient and hydration status (Livneh et al., 2017, 2020). On-going insular cortex activity was demonstrated to reflect current bodily status – which was not dependent on hunger or thirst neuronal activity (Livneh et al., 2020). These results suggest the insular cortex may be a key region involved in the physiological response to visceral or sensory stimuli that may not accurately represent current homeostatic state – especially in conditions of obesity or anorexia.

Human imaging studies indicate that the insular cortex is disrupted in obesity (Frank et al., 2013; Mata et al., 2015). Insular cortex activity in obese adolescents correlated with external eating (eating in response to external cues), and was inversely associated with interoceptive sensitivity, the opposite pattern from that observed in healthy weight adolescents (Mata et al., 2015). This preferential tuning of the insular cortex, to external rather than internal cues in obesity could explain why obese individuals consume food well beyond their metabolic needs. In response to food cues obese adults show higher insular cortex, BOLD activation as well as increased functional connectivity to the ACC compared to normal weight adults (Mata et al., 2015). However, human imaging studies also show the insular cortex is involved in anorexia, where it responds to negative emotional associations patients have with high-calorie foods (Ellison et al., 1998a; Uher et al., 2004). Given that the insular cortex is important for sensing internal cues of hunger or satiety and external food-related cues such as taste or pleasurable properties of a food, the functional connections between the insular cortex, and LH may mediate the balance between hedonic and homeostatic food intake. Recently, a key study by Wu *et al* (2020) demonstrated that the right anterior insular cortex responds to aversive visceral signals, and that neurons from this region project to LH^{Vglut2} neurons to suppress food intake and body weight (Wu et al., 2020). This study suggests that hyper-activation of the insular to LH circuit may be a factor underlying anorexia, and provides a strong rationale for investigating other cortical to LH circuits.

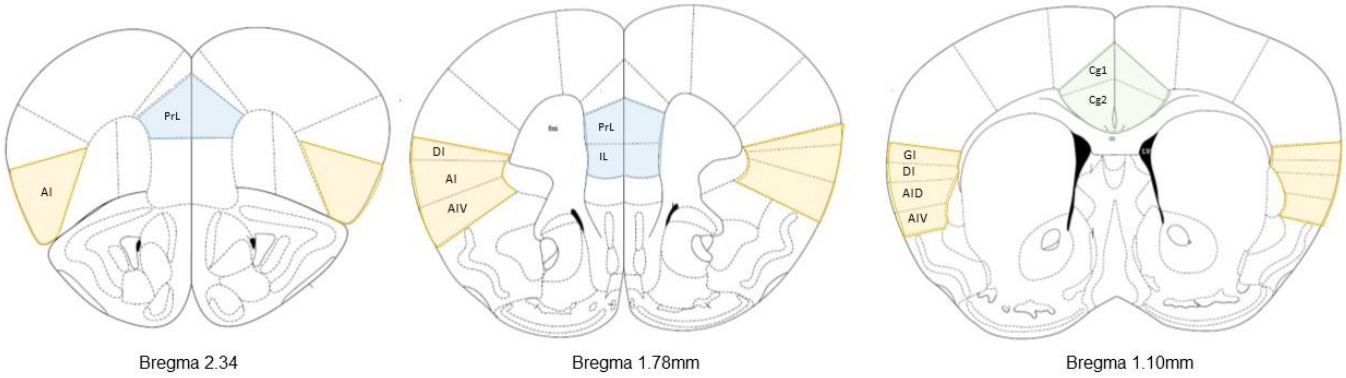

Figure 1.2. Coronal sections of the mouse brain highlighting the cortical regions involved in the control of food intake. Yellow = Insular Cortex; Blue = Medial pre-frontal cortex; Green = Anterior cingulate cortex. Abbrieviations: AI – Agranular Insula Cortex; AID – Agranular Insula Cortex Dorsal Part; AID – Agranular Insula Cortex Ventral Part; Cg1 - Cingulate gyrus 1; Cg2 – Cingulate gyrus; DI - Disgranular Insular Cortex; GI – Granular Insular Cortex; IL – Infralimbic Cortex; PrL – Prelimbic cortex. Adapted from Allen Brain Atlas.

1.6 Homeostatic control of food intake and behaviour

The homeostatic control of food intake is far better understood than the non-homeostatic drive to eat. However, recent studies show that hunger responsive neurons do much more than simply promote food consumption. Within the hypothalamus, the ARC is particularly important in responding to fluctuations in nutrient status. Agrp and POMC neurons discussed previously mediate reponse to hunger and satiey – both behaviouraly and physiologically. Agrp neruons are important for regulating appropriate energy intake: ablation of the Agrp neurons in adult mice results in starvation and death (Gropp et al., 2005; Luquet et al., 2005) while activation of these neurons using either chemogenetics (Krashes et al., 2011) or optogenetics (Aponte et al., 2011) induces feeding in sated animals. However, several studies have reported that Agrp activation facilitates adaptive behaviours in the absence of food; promoting foraging behaviours (Dietrich et al., 2012); reducing anxiety and territorial aggression (Padilla et al., 2016); suppressing innate competing drives such as social interaction and thirst (Burnett et al., 2016); and suppressing pain (Alhadeff et al., 2018). Occurring simultaneously these behaviours would be advantageous to a hungry animal who may need to leave a familiar environment in order to locate food. Agrp neuronal activity is aversive in the absence of food (Betley et al., 2015), and is rapidly suppressed upon the sight or smell of food (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015). This negative reinforcement has been proposed to drive the coordination of diverse food-seeking behaviours (Betley et al., 2015).

The mechanisms by which Agrp neurons drive adaptive behaviour is not yet fully understood. Agrp neurons project to their targets in what appears to be a one-to-one manner with minimal collateralisation, and activation of isolated circuits does not always evoke a strong feeding response (Betley et al., 2013). For example, activation of Agrp to the medial nucleus of the amygdala (MeA) pathway is sufficient to suppress competing drives, such as territorial behaviour but does not stimulate feeding to the same extent as the activation of all Agrp circuits (Padilla et al., 2016). Whereas activation of Agrp terminals in the paraventricular nucleus of the hypothalamus (PVH) results in a similar level of food intake to activation of all Agrp circuits (Betley et al., 2013), this circuit does not affect territorial aggression (Padilla et al., 2016). Clearly, there exists

distinct subpopulations of Agrp neurons that play various and sometimes redundant roles in coordinating the behavioural response to hunger.

The seemingly contradictory observations that artificial Agrp activation drives feeding (Aponte et al., 2011; Krashes et al., 2011), yet naturally, Agrp activity is rapidly inhibited upon the presentation of food or associated cues (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015) can be resolved by considering the long-lasting effect of Agrp neuronal activity. A key study by Chen et al. 2016 demonstrated that optogenetic stimulation of Agrp neurons prior to the presentation of food is sufficient to induce a strong feeding response, comparable to that seen following an overnight fast (Chen et al., 2016). The response seen, measured as food intake, appears to be proportional to the duration of the pre-stimulation period, up until a 30 minute pre-stimulation period where the response then begins to plateau (Chen et al., 2016). Supporting the idea that pre-emptive Agrp neuronal activity coordinates a sustained hunger response, Jikomes et al. 2016 found that mice with stimulation of Agrp neurons prior to entering a foot-shock arena, endured more shocks to receive a food reward than mice with neuronal stimulation restricted to time in arena only (Jikomes et al., 2016). Clearly Agrp signalling over a period of time will affect behaviours related to feeding and food –seeking. These factors are important to consider when investigating both homeostatic and non-homeostatic feeding and behaviour, and are relevant to the experimental chapters presented herein.

1.8 Conclusion

Clearly, the LH is ideally positioned to integrate metabolic signals with environmental and sensory information. Our understanding of this complex brain region has advanced rapidly over the past decade due to the variety of new tools and techniques now available in neuroscience. However, how the LH balances homeostatic and hedonic inputs to coordinate feeding and motivated behaviour has not yet been fully elucidated. The cortical regions of the brain including the mPFC, ACC, and insular cortex are responsible for reward processing, decision-making and top-down control of behaviour. The reciprocal connections between the LH and these cortical regions are therefore likely to be important in the decision of what and when we eat. In obesity, metabolic sensing mechanisms are disrupted and environmental food cues take precedence. Human studies show that the functional connectivity between cortical regions and the hypothalamus is perturbed in obesity, yet so far there have not been any mechanistic studies into the relevance of this observation for the development or maintenance of the disease. Systematically determining the functional significance of the circuits between the LH and the mPFC and ACC important in advancing our understanding of how the brain controls food intake. In addition to this, there is recent evidence to suggest that hunger sensing Agrp neurons promote adaptive behaviours as part of their role in increasing food intake. However, whether these neurons are able to promote adaptive behaviours in the face of stress is not yet clear.

1.9 Project aims and methodological approach

Broadly, this project aims to apply cutting-edge techniques to characterise the neurons and neural circuits involved in feeding and food seeking behaviours. Two separate ideas are investigated in the thesis: 1) how

homeostatic circuits can influence behaviours beyond simply food consumption (Chapter 2); and 2) how non-homeostatic circuits may act to override metabolic cues and potentially lead to disordered eating (Chapters 3&4). Specifically, chapter 2 aims to investigate the role of the hunger sensing Agrp neurons in hormonal and behavioural response to an acute stressor. Chapters 3 and 4 aim to investigate the role of cortical inputs to the LH identified in human obesity studies (mPFC and ACC respectively) in the control of food intake and reward-seeking behaviour. The chapters are formatted for publication and therefore stand alone, each with a specific methods section. However, the methodology used across the three chapters is largely overlapping and relies on the use of viral tools to target specific neurons based on gene expression or projection region. In chapter 2, we utilise transgenic Agrp-cre expressing mice to allow us to remotely activate these neurons using either chemogenetic or wireless optogenetic technology. Both of these techniques enable acute manipulations of the Agrp neurons, and allow for observation of associated behaviours in freely behaving animals. In Chapters 3 and 4, a retrograde virus is used in C57BL/6 mice to deliver cre-recombinase specifically to the mPFC to LH projection neurons and ACC to LH projection neurons respectively. This then allows a second cre-dependent virus to be targeted to the projection region of interest, essentially enabling this circuit to again be remotely manipulated. In chapters 3 and 4, in addition to the chemogenetic and wireless optogenetic technology used in chapter 2, we also utilise a genetically engineered caspase virus to chronically ablate the mPFC to LH circuit and the ACC to LH circuit. Together these techniques allow us to infer the effects of activation and chronic inhibition of these circuits on feeding and reward seeking behaviours. In chapter 5, the findings from the 3 experimental studies are discussed together, along with strengths and weaknesses of the techniques utilised and future directions of the research.

2. Chapter Two: Agrp neurons enable adaptive responses to acute stress to promote food seeking

Agrp neurons enable adaptive responses to acute stress to promote food seeking.

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2.1 Abstract

Hunger is a complex physiological drive that affects both the mood and motivation of an organism in order to promote food consumption and ultimately restore energy balance. Agouti-related peptide (Agrp) neurons in the hypothalamus sense hunger and promote feeding, however when food is unavailable, Agrp neurons promote adaptive behaviors by reducing anxiety and increasing food-seeking behavior. A fundamental question remains; what are the physiological mechanisms through which Agrp neurons regulate adaptive behaviors. In this study, we examined whether Agrp neurons engage the Hypothalamic Pituitary Adrenal (HPA) stress axis to control stress reactivity and adaptive anxiolytic behaviors. Activation of Agrp neurons using a hM3Dq DREADD approach prior to acute restraint stress reduced subsequent anxiety-like behavior, increased memory recall and promoted food-seeking and food intake. Prior activation of Agrp neurons before acute restraint stress significantly increased plasma corticosterone (CORT) 15, 30 and 60 minutes and ACTH 30 minutes after stress onset. Anterograde tracing of Agrp neurons using the cre-dependent herpes simplex virus H129 ΔTK-TT confirmed that Agrp neurons target ~30% of CRH neurons in the PVN. Pretreatment with the CORT biosynthesis inhibitor Metyrapone indicated that an increase in plasma CORT is not required for Agrp-mediated adaptive behaviors following stress. Activation of Agrp neurons only during the 15-minute restraint stress, using a novel wireless optogenetic approach, was not sufficient for the full expression of adaptive behavioural responses observed following prolonged Agrp hM3Dq DREADD activation. Our results suggest prolonged Agrp neuronal activation, not just during an acute stressor, is required to mitigate anxiety and improve memory recall after acute stress. Moreover, although Agrp increase CORT through the HPA axis in response to acute stress, this is not required to affect adaptive behavioural responses. Thus, Agrp neurons are uniquely positioned to integrate metabolic information with mood and memory function to optimize current food-seeking and future food-seeking opportunities in an acutely stressful dangerous environment.

2.2 Introduction

Feeding is a fundamental requirement for homeostasis and survival. As such, appropriate hunger-sensing during energy deficit is essential to regulate numerous homeostatic biological processes including reproduction, growth and repair. In the case of energy homeostasis, agouti-related peptide (Agrp) neurons, which co-express neuropeptide Y (NPY) and GABA, in the hypothalamic arcuate nucleus (ARC) are crucial to respond to a state of hunger or energy deficit by promoting energy intake and energy conservation. For example, ablation of these neurons in adult mice results in starvation (Gropp et al., 2005; Luquet et al., 2005) while activation of these neurons using either chemogenetics (Krashes et al., 2011) or optogenetics (Aponte et al., 2011) induces feeding in sated animals. More recently, several studies have reported that Agrp neuron activation facilitates adaptive behaviors in the absence of food; promoting foraging and exploratory behaviors (Dietrich et al., 2012, 2015); reducing territorial aggression (Padilla et al., 2016); suppressing innate competing drives such as social interaction and thirst (Burnett et al., 2016); and suppressing pain (Alhadeff et al., 2018). These adaptive behaviors and responses would be advantageous to a hungry animal who may need to leave a familiar or safe environment in order to locate food. Intriguingly, high Agrp neural activity promotes aversive learning in the absence of food (Betley et al., 2015) and this negative reinforcement may be a key driver through which Agrp neural activity coordinates a diverse range of adaptive behaviors, beneficial to food-seeking. In support of this notion, the presentation (sight or smell) of food rapidly suppresses Agrp neural activity (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015), which would immediately remove this aversive signalling. Indeed, Agrp neural activity will return to a higher state if food is caged and unable to be consumed or removed before food consumption leads to satiation (Chen et al., 2015). Another interpretation from the seemingly contradictory observations that artificial Agrp activation drives feeding (Aponte et al., 2011; Krashes et al., 2011), yet naturally, Agrp activity is rapidly inhibited upon the presentation of food or associated cues (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015) can be resolved by considering the long-lasting effect of Agrp neural activity to convey a hunger signal and initiate a feeding response (Chen & Knight, 2016). An important study by Chen et al. 2016 demonstrated that optogenetic stimulation of Agrp neurons only prior to the presentation of food is sufficient to induce a strong feeding response once food is returned, comparable to that seen following an overnight fast . The feeding response is proportional to the duration of the pre-stimulation period, up until a 30 minute pre-stimulation period where the response then begins to plateau (Chen et al., 2016). In addition, Jikomes et al. 2016 found that mice with stimulation of Agrp neurons prior to entering a foot-shock arena, endured more shocks to receive a food reward than mice with neuronal stimulation restricted to the time in arena only (Jikomes et al., 2016). Collectively these studies support the idea that Agrp neuronal activity coordinates a sustained hunger response, which supports a feeding response after activation is terminated, and this prior activation engages adaptive behaviors to facilitate food seeking and food retrieval.

Food-seeking during periods of hunger comes with inherent risks for prey species, such as foraging in unfamiliar environments with a greater risk of predation. Therefore, hungry animals are likely to be exposed to more acute stressors when food-seeking. Based on evidence that Agrp stimulation or fasting reduces

anxiety and territorial aggression (Dietrich et al., 2015; Padilla et al., 2016), we reasoned Agrp hunger-signalling would attenuate the effects of acute stressors and produce a range of adaptive behaviors, such as reduced anxiety and greater exploration of a novel environment, to optimise and prioritise food-seeking.

The hypothalamic-pituitary-adrenal axis (HPA) axis is the principal neuroendocrine stress pathway with corticotrophin-releasing-hormone (CRH) neurons in the paraventricular nucleus (PVN) initiating the HPA response to stress, inducing the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary and subsequent release of corticosterone (CORT) from the adrenal cortex. Agrp neurons express glucocorticoid receptors and recent studies demonstrate Agrp neurons are activated by CORT (Perry et al., 2019) . In addition, it is well known that fasting and starvation activate the endocrine arm of the HPA axis (Dallman et al., 1999). A number of older studies also show that NPY injection in the PVN increases *Crh* mRNA, immunoreactivity, and peptide release (Haas & George, 1987; Suda et al., 1993; Tsagarakis et al., 1989). This is supported by the observation that acute fasting (6 hours) also increases *Crh* mRNA levels (Dallman et al., 1999). Given that NPY is co-expressed with Agrp neurons in the ARC, these studies offer a potential link between hunger-sensing Agrp/NPY neurons in the ARC and the HPA axis. Moreover, NPY confers an anxiolytic-like behavioral response (Das & Patri, 2017; Desai et al., 2014; Sabban et al., 2015), and antagonising the NPY5 receptor attenuates adaptive food seeking behaviors (Dietrich et al., 2015). Currently it is unknown whether Agrp neurons influence the responsiveness of HPA axis to an acute stressor and whether this drives stress-induced behavioral changes. We hypothesized that activation of Agrp neurons potentiates a HPA CORT response to actue restraint stress and mitigates restraint stress-induced anxiety to promote the expression of a range of behaviors that prioritise food-seeking and consumption of food.

2.3 Methodology

Animals

All experiments were conducted in accordance with the Monash Animal Ethics Committee guidelines. Agrp-ires-*cre* mice on a C57BL/6 background were obtained from Jackson Laboratory Agrp$^{tm1(cre)Low/J}$ (stock no. 012899) and crossed with NPY GFP mice (B6.FVB-Tg(Npy-hr*GFP*)1Lowl/J; stock number 006417; The Jackson Laboratory, Maine, USA) and bred in the Monash Animal Services facility. For clozapine and clozapine-n-oxide (CNO) experiments C57/BL6 mice were obtained from Monash Animal Services facility. Mice were maintained on a 12-hour light-dark cycle (7am-7pm) with *ad libitum* access to standard chow and water under standard laboratory conditions (21°C). All mice were individually housed following surgery and all experiments were conducted midway through the light phase (9am-3pm) to minimise any influence on behaviour an hormones due to circadian rhythm. Mice were handled for 5 min each day for five consecutive days leading up to stress response and behavior experiments.

Viruses and Surgical Procedures

Mice were anesthetised using isoflurane (5% for induction, 2% for maintenance) and positioned on a stereotaxic frame (Stoelting). A Neuros syringe (Hamilton, Reno, NV, USA) was used to deliver virus to the target regions; ARC (-1.7mm Bregma; +/- 0.2 mm lateral; -5.6mm ventral from surface of brain). To transduce stimulatory DREADDs into the Agrp neurons a cre-dependent viral construct was used (AAV5-DIO-hM3Dq-mCherry; Fig 1A; UNC vector core). DREADD viruses were injected in volumes of 200 nL bilaterally. For viral tracing studies the cre-dependent anterograde trans-synaptic tracer H129 strain of the *Herpes simplex* virus was used (HSV1 H129 ΔTK-TT) and injected unilaterally at a volume of 200 nL in Agrp-ires-*cre* mice. HSV1 H129 ΔTK-TT was produced by homologous recombination of HTK targeting vector (generously provided by David Anderson Division of Biology, California Institute of Technology, USA) with wildtype HSV1 H129, as previously described (Lo & Anderson, 2011). For optogenetics experiments a cre-dependent viral construct was injected (AAV-DJ EF1a-DIO-hChR2(H134R)-mCherry; Fig 5A; Stanford Gene and Viral Vector Core) 200nL bilaterally. A wireless antenna (Fig 5A; 9.8mm diameter, 1.3mm thickness, 6mm probe length; 30 mg weight; Neurolux) was then implanted above the arcuate nucleus unilaterally and fixed to the skull of the mouse using the procedure described in Shin *et al* 2017 (Shin et al., 2017).

Immunohistochemistry

For c-fos experiments, mice were injected with saline or Clozapine-N-oxide (CNO) (0.3mg/kg i.p.; Sigma) and 60 minutes later were deeply anesthetised and then transcardially perfused with 0.05 M phosphate buffered (PB) saline followed by 4% *w/v* paraformaldehyde (PFA) in 0.1 M PB. Brains were post-fixed in 4% PFA in 0.1 M PB overnight before being transferred to 30% sucrose solutions. Coronal sections of the whole brain were cut at 30 μm in sets of 4. A 1 in 4 series of tissue sections was washed in 0.1 M PB 3 times before being blocked in 1% H_2O_2 in 0.1 M PB for 15 min and then washed again. Tissue was then placed in 0.3% Triton X-100 in 0.1 M PB and 4% normal horse serum for 1-hour before an overnight incubation at 4°C in primary antibody: anti-CRH (rabbit, 1:1000, provided by the late Professor Wylie Vale), anti-mCherry

(chicken, 1:1000, Abcam), anti-c-fos (rabbit, 1:1000, Santa-Cruz Biotechnology). Tissue sections were then washed again before a 90 min incubation in secondary antibody at room temperature; goat anti-rabbit IgG AlexaFluor 488 (1:400; Life Technologies), goat anti-chicken IgG AlexaFluor 594 (1:400, Life Technologies). The sections were then mounted and coverslipped with hard set mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) (Vector) used to counterstain DNA.

Food intake and behavioral analysis in response to acute stress

Food intake in home cage

For DREADD studies, mice were injected with CNO (1mg/kg i.p.; Sigma) or saline (0.9% 10mL/kg i.p.) and returned to their home cages without food for 3 hours. Then either food was returned, or mice were placed in restraint stress for 15 minutes and then food was returned, and food intake was measured 2, 4 and 24 hours later per mouse (5, 7 and 27 hours after CNO injection). For restraint stress, mice were placed into 50 mL plastic Falcon® tubes that were modified to include holes throughout for ventilation. Mice were confined to tubes for 15 minutes. For Clozapine studies, Clozapine (0.01mg/kg i.p.; Sigma) or saline (0.9% 10mL/kg i.p.) was administered to C57/BL6 mice as controls. For metyrapone studies, WT and Cre expressing animals were injected with CNO (1mg/kg i.p.; Sigma) and metyrapone (75mg/kg i.p.; Sigma) prior to experimentation.

Anxiety-like behavior following stress

Mice were injected with CNO (1mg/kg i.p.) or saline (0.9% 10mL/kg i.p.) and returned to their home cages without food for 3 hours before being placed in restraint stress for 15 minutes. For Clozapine studies, Clozapine (0.01mg/kg i.p.; Sigma) or saline (0.9% 10mL/kg i.p.) were injected in C57/BL6 mice as controls. For metyrapone studies, WT and Cre expressing animals, previously injected with DREADDs were injected with CNO (1mg/kg i.p.; Sigma) and metyrapone (75mg/kg i.p.; Sigma) prior to experimentation. For these experiments Agrp WT mice injected with CNO where used as controls for Agrp-ires-cre mice injected with CNO. We used a 3 hour activation time, without food available, before stress since previous work demonstrates Agrp neurons must already be active to initiate cued food-seeking behavior in a threatening arena (15) and Agrp neuronal activation manifests different behavioral outcomes without food availability (6), reflecting heightened hunger-signaling in the brain. Immediately following stress, mice were placed into 1of 3 behavioral tests (detailed below) for 6 minutes and behaviors filmed. Videos were analysed using software (Top Scan Lite, Clever Sys).

Elevated plus maze

The elevated plus maze (400 mm above floor) consisted of two open arms (310 mm X 50 mm) and two closed arms (310 mm X 50 mm with 150 mm high barriers). Mice were placed in the centre of the plus maze facing an open arm and allowed to explore the apparatus for the duration of the test (6 minutes). The time spent exploring both open arms was measured. The time spent in the centre of the plus maze was excluded from analysis unless otherwise stated.

Light dark emergence test

The light-dark box (285 mm wall height) was constructed from wood and consisted of a larger light chamber (480 mm X 295 mm; painted white) and a smaller dark chamber (150 mm X 295 mm; painted black) separated by a small opening. Mice were placed in the centre of the dark chamber and allowed to move freely about the two compartments for the duration of the test (6 minutes). The time the mouse spent in the light chamber was measured.

Large open field

Mice were placed in the outer ring of a large open field (800 mm diameter with wall height 300 mm) and allowed to freely move about the apparatus for the duration of the test (6 minutes). Time spent in the centre zone (200 mm diameter), and distance moved were measured.

Food intake in anxiogenic novel environment

This experiment was designed to test whether Agrp activation promoted food seeking and feeding behavior in a novel environment, since novel environments are known to induce acute stress and anxiety in rodents (Day et al., 2001; Frédéric et al., 2006). Mice were injected with CNO (1mg/kg i.p.; Sigma) or saline (for metyrapone studies Agrp WT and CRE were both injected with CNO (1mg/kg i.p.; Sigma) and metyrapone (75mg/kg i.p.; Sigma)) and immediately returned to their home cages without food for 3 hours to produce a heightened prolonged sense of hunger, rather than just an immediate feeding cue, before being placed into the novel environment, either open field or light dark box baited with food. Moreover, prior activation of Agrp neurons is required to initiate cued food-seeking behavior in a threatening arena (Jikomes et al., 2016), highlighting the potential differences in behavioral outcomes with immediate vs pre-emptive Agrp activation. Separate cohorts of mice were used here to avoid familiarization to the apparatus. Two pellets of standard chow (4.2-4.8 g in total) were placed in a small porcelain dish (70 mm diameter) in the food zone (100mm diameter), which was in the centre of the open field or in the centre of the light chamber of the light-dark box (Fig 3A, E). Mice were placed in the baited apparatus for 30 minutes and food intake and time spent in the food zone was measured.

Novel object recognition task

A modified version of a standard novel object recognition protocol (Bevins & Besheer, 2006) was used (Fig 2J). Mice were habituated to the empty novel object chamber (200 mm X 350 mm, with wall height 350 mm) the day before the experiment for 10 minutes. On the day of the experiment mice were injected with CNO (1mg/kg; Sigma) or saline (or for metyrapone studies Agrp WT and CRE were both injected with CNO (1mg/kg; Sigma) and metyrapone (75mg/kg; Sigma)) 2.5 hours before being placed into the novel field chamber which contained two identical to-be familiarised-objects (either two blue tube lids or two clear 5ml vials with yellow lids). The familiar and novel objects were validated to be equally interesting to C57BL/6 mice and were counter-balanced in the experiment. The mice were given 10 minutes to interact with the objects

before being returned to their home cages for 20 minutes. Mice were then placed in restraint stress for 15 minutes and immediately returned to the novel object chamber, this time with one of the familiar objects replaced with the novel object. Mice were given 2 minutes to interact with the objects and the time the mouse spent near to each object (within a 10 mm radius) was measured.

Optogenetic stimulation of Agrp neurons

For all optogenetic experiments light stimulation was delivered at 20Hz, 3 seconds on 1 second off, using wireless LED probes (Fig5A; Neurolux), which was inserted using the surgical procedures described above. An electromagnetic field was constructed around an animal cage (440mm X 340mm with wall height 200mm) and connected to a power distribution control box (12Amps; 10W; Neurolux) which delivered radio frequency power to the wireless LED probes. Prior to each experiment the antenna was tuned to the power distribution control box to ensure the impedance of the power source and antenna matched. Each antenna was tuned to ensure a standing wave ratio of <1.3 and an impedance of 50Ω. Blue light delivery (470nm) was in the range of 1-5mW. The frequency of stimulation was based on data showing Agrp neurons in the fasted state fire as high as 20Hz in vivo (Mandelblat-Cerf et al., 2015), and previous optogenetic studies (Aponte et al., 2011). For the food intake experiment, mice were given access to chow during the light cycle and food intake was measured in their home cage 1-hour before, during and 1-hour post optogenetic stimulation.

Real-time place preference

Mice were given 10 minutes to explore the real-time place preference apparatus (385mm X 245mm with wall height 170mm) before 20 minutes of stimulation (20Hz, 3 seconds on, 1 second off) only one side of the apparatus (Fig 5D). Preference for stimulation period was calculated before and during stimulation using behavioral tracking software (EthoVision XT version 14.0.1322, Noldus Information Technology).

HPA-axis response to stress

For DREADD studies, mice were injected with CNO (1mg/kg i.p.) or saline 3 hours before being subjected to restraint stress. For metyrapone studies mice were injected with both CNO (1mg/kg; Sigma) and Metyrapone (75mg/kg; Sigma) 3 hours before being subjected to restraint stress. For optogenetics studies, light stimulation was delivered only during 15-minute restraint stress period. On the day of experimentation, previously handled mice were brought into the testing room at 7 am and allowed 4 hours to acclimatize to the experimental room. Tail vein blood samples were collected immediately before and after 15 minutes of restraint stress, and then at intervals of 30, 60, 90 and 120 minutes following stress. CORT levels were assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Abnova, Heidelberg, Germany) according to manufacturer's instructions, with an intra-assay and inter-assay variability <8% coefficient of variation (CV) and the lowest level of detection at 110 pg/mL. To measure adrenocorticotropic hormone (ACTH) a separate cohort of mice was placed in restraint stress for 15 minutes and 30 minutes after restraint stress onset, mice were deeply anesthetised and decapitated and trunk blood was collected. ACTH levels were assayed using an ELISA kit (MD Biosciences, St Paul, MN, USA) according

to the manufacturer's instructions with an intra-assay variability of 3.1-4.2% CV, inter-assay variability of 5.8-6.2% and the lowest level of detection at 0.46 pg/mL.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism for MacOS X (version 7.0b). All data are represented as mean ± standard error of the mean (SEM). Two-way repeated measures ANOVA with *post hoc* tests were used to determine statistical significance between treatment and time for CORT. A two-tailed Student's unpaired *t*-test was used when comparing saline vs CNO only. $p<0.05$ was considered statistically significant. Pearson's correlation was used to compute the relationship between food intake during optogenetic stimulation and behavior in the elevated plus maze.

2.4 Results

Agrp DREADD validation.

We confirmed hM3Dq DREADD expression by visualizing the expression of mCherry reporter in the ARC after all testing in all experimental animals. The mCherry reporter expression was confined to the ARC (Fig 1B, middle) and co-expressed in NPY GFP neurons (Fig 1B, right), suggesting selectively targeted hM3Dq DREADD expression to Agrp neurons, as approximately 90% of NPY GFP neurons coexpress Agrp (Betley et al., 2013). To functionally validate the stimulatory DREADD expression in Agrp neurons, food intake and c-fos expression were analysed in Agrp-ires-*cre*::NPY GFP mice. Intraperitoneal CNO injection increased food intake after 2 hours (Fig 1C) and c-fos activation in identified NPY GFP neurons after 30 minutes (Fig 1D&E) compared to saline-injected controls, indicating functional activation of Agrp neurons expressing the hM3Dq DREADD.

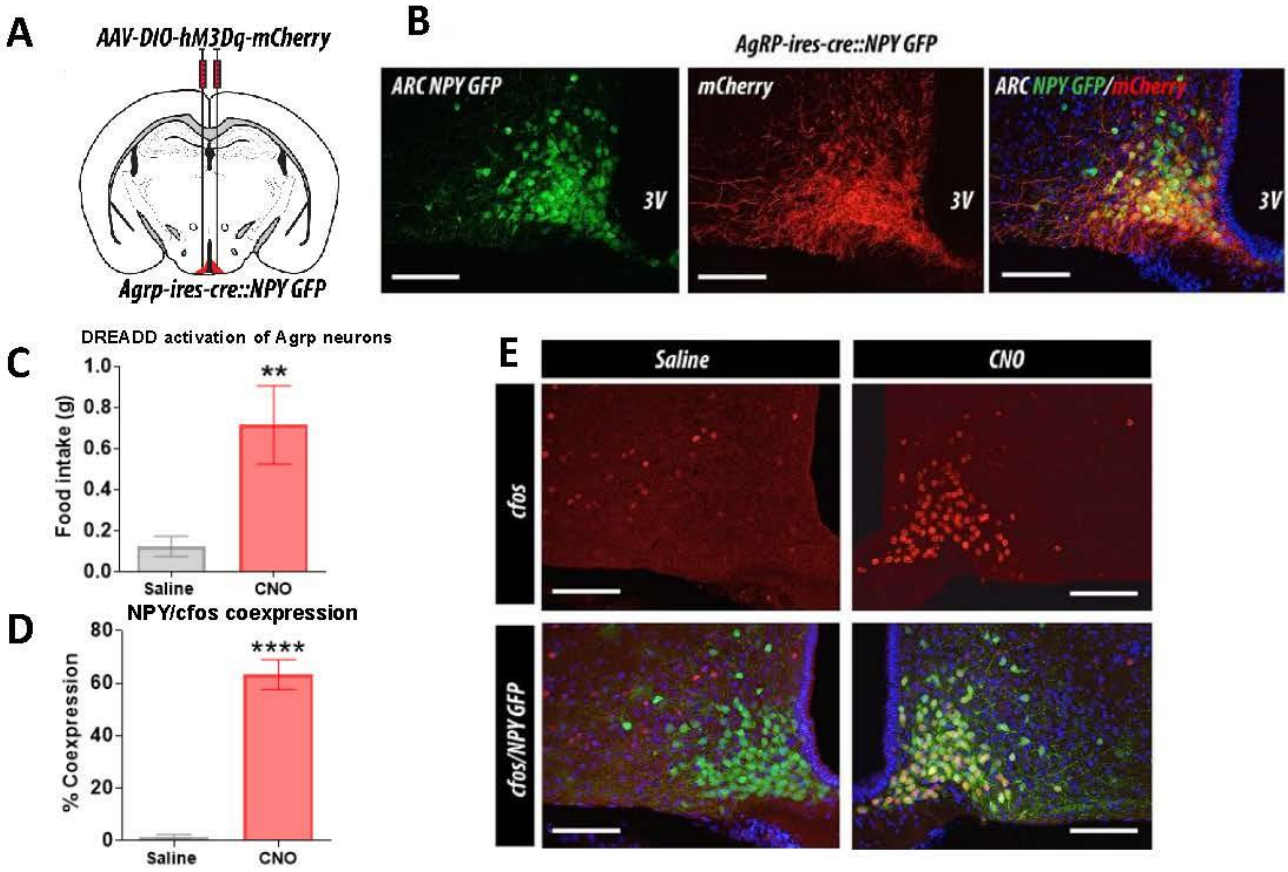


Figure 1 – DREADD validation in Agrp neurons. The stimulatory DREADD, AAV-DIO-hM3Dq-mCherry, was injected bilaterally into the ARC nucleus of Agrp-ires-cre::NPY GFP mice (A). Representative image showing NPY GFP expression, Agrp cre-dependent AAV-DIO-hM3Dq-mCherry expression and co-expression between NPY GFP and Agrp cre-dependent AAV-DIO-hM3Dq-mCherry neurons (B). Activation of Agrp neurons increases food intake 2 hours after CNO injection (C; n=13, Student's unpaired t-test). CNO injection increases c-fos activation, compared to saline, 30 minutes later in identified NPY GFP positive neurons (D; n=6-7; Student's unpaired t-test). Representative CNO-induced c-fos activation in identified NPY GFP positive neurons (E). Data are expressed as mean ± sem and ** indicates $p<0.01$, **** indicates $p<0.0001$. Scale bars represent 60 µm.

Agrp activation regulates feeding and anxiety-like behavior following acute stress.

Prolonged hunger necessitates animals to take greater risks to seek food. We reasoned that prolonged Agrp neural activity enables mice to cope with acute stressors by enacting adaptive behaviors, such as reduced anxiety, to help promote food seeking and consumption. To test if Agrp activation promotes food-seeking and food intake in the face of acute stress, we activated Agrp neurons for 3 hours in the absence of food prior to restraint stress and then examined food intake after restraint stress (Fig 3A-B). We chose 3 hours of activation as we hypothesized that prolonged hunger signalling is required to maximize expression of adaptive behaviors. Furthermore, previous research shows differential behavioral responses to pre-emptive vs simultaneous Agrp stimulation in cued food-seeking in a threatening arena. We used a 15 minute restraint stress period as this produces greatest hormonal and neural responses to stress compared to longer restraint stress periods (Weinberg et al., 2007). Our results indicate that DREADD-induced Agrp activation significantly increased food intake 2 hours after restraint stress, (5 hours after CNO injection; Fig 2C). Although a trend of p=0.069 was seen at 4 hours after restraint stress (7 hours after CNO), there were no significant differences at 4 or 24 hours after restraint stress (Fig 2D&E).

Our results show that prior activation of Agrp neurons also significantly increased the time mice spent in the open arm of the elevated plus maze (Fig 2G) and the light zone of the light-dark box (Fig 2H). Consistent with other results demonstrating Agrp activity increases locomotor activity (Krashes et al., 2011; Padilla et al., 2016), Agrp activation prior to acute restraint stress increased distance moved in the open field (Fig 2I). Next, we tested if prior activation of Agrp neurons affected memory recall after acute stress using a novel object recognition test (Fig 2J). After acute restraint stress, mice with prior activation of Agrp neurons spent significantly more time with a novel object suggesting greater memory recall (Fig 2K) but no differences in total object interaction time (Fig 2J). Importantly, we tested the effects of CNO versus saline in C57/BL6 mice without viral transduction, as additional controls to determine whether CNO (1 mg/kg) had any non-specific effects on food intake or light-dark box and elevated plus maze behavior. We saw no differences between CNO and saline in any of the experimental parameters measured (Sup Fig 1A-F). We also tested the effects of clozapine, the major metabolite of CNO on food intake and EPM, light-dark box and novel object behavior and again found no differences between saline and clozapine (0.01mg/kg; Sigma) (Sup Fig 2A-F). These important additional controls help to strengthen our conclusions that CNO-induced DREADD activation of Agrp neurons, not off-target effects of CNO or clozapine, reduce anxiety and improve memory recall after acute restraint stress.

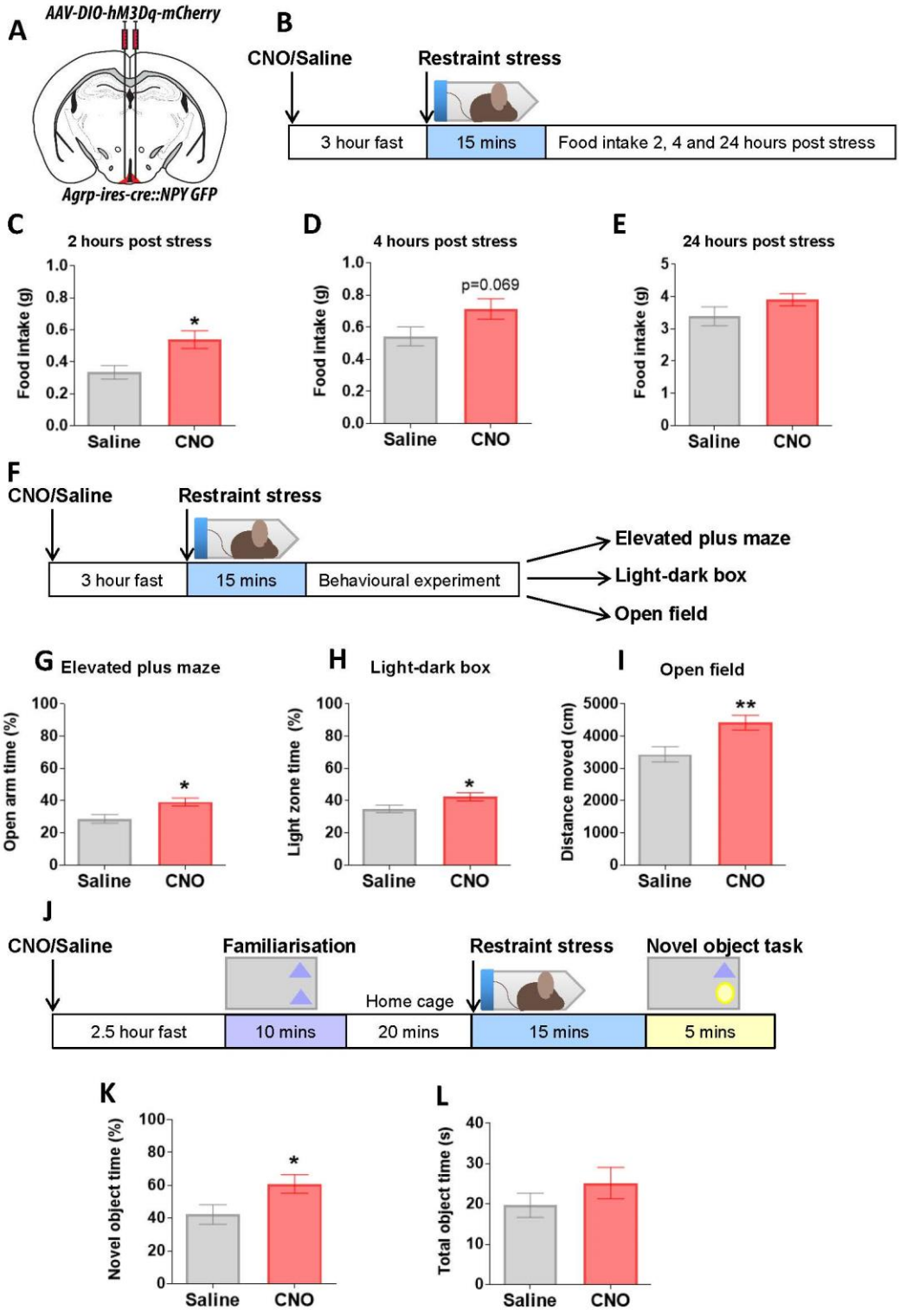


Figure 2 – Agrp activation regulates behavior responses to acute restraint stress. The stimulatory DREADD, AAV-DIO-hM3Dq-mCherry, was injected bilaterally into the ARC nucleus of Agrp-ires-cre::NPY GFP mice (A). Experimental approach for feeding behavior experiment (B). CNO-induced (1mg/kg) food intake at 2 hours (5 hours post CNO), 4 hours (7 hours post CNO), and 24 (27 hours post CNO) hours after restraint stress (C-E; n=10-13, Student's unpaired t-test). Experimental approach for anxiety-like behavior experiments (F). CNO increases the time spent in the open arm of the elevated plus maze (G; n=10-17; Student's unpaired t-test) and the time spent in the light zone of the light-dark box (H; n=20-31; Student's unpaired t-test), as well as increases distanced moved in the open field (I; n=10-17). Experimental approach for novel object recognition (J). CNO increases time spent investigating the novel object (K, n=11, student's

t-test) but does not affect total object interaction time (L, n=11, student's t-test). Data are expressed as mean ± sem and * indicates p<0.05, ** indicates p<0.01.

Agrp activation promotes feeding in novel environments.

Given that exposure to novel environments produces an acute stress response and anxiety in mice (Day et al., 2001; Frédéric et al., 2006), we next asked whether prior activation of Agrp neurons would help promote food seeking and food intake in a novel environment (Fig. 3 A&E). In these experiments, mice were not previously exposed to the open field or light-box dark to prevent acclimatisation to behavioral apparatuses. In both the open field and light-dark box, prior activation of Agrp neurons increased time in the food zone and 30-minute food intake in the novel environment (Fig 3B, C, F, G), indicating that prior activation of Agrp focuses mice on the salient task of finding food in anxiety-promoting and novel environments.

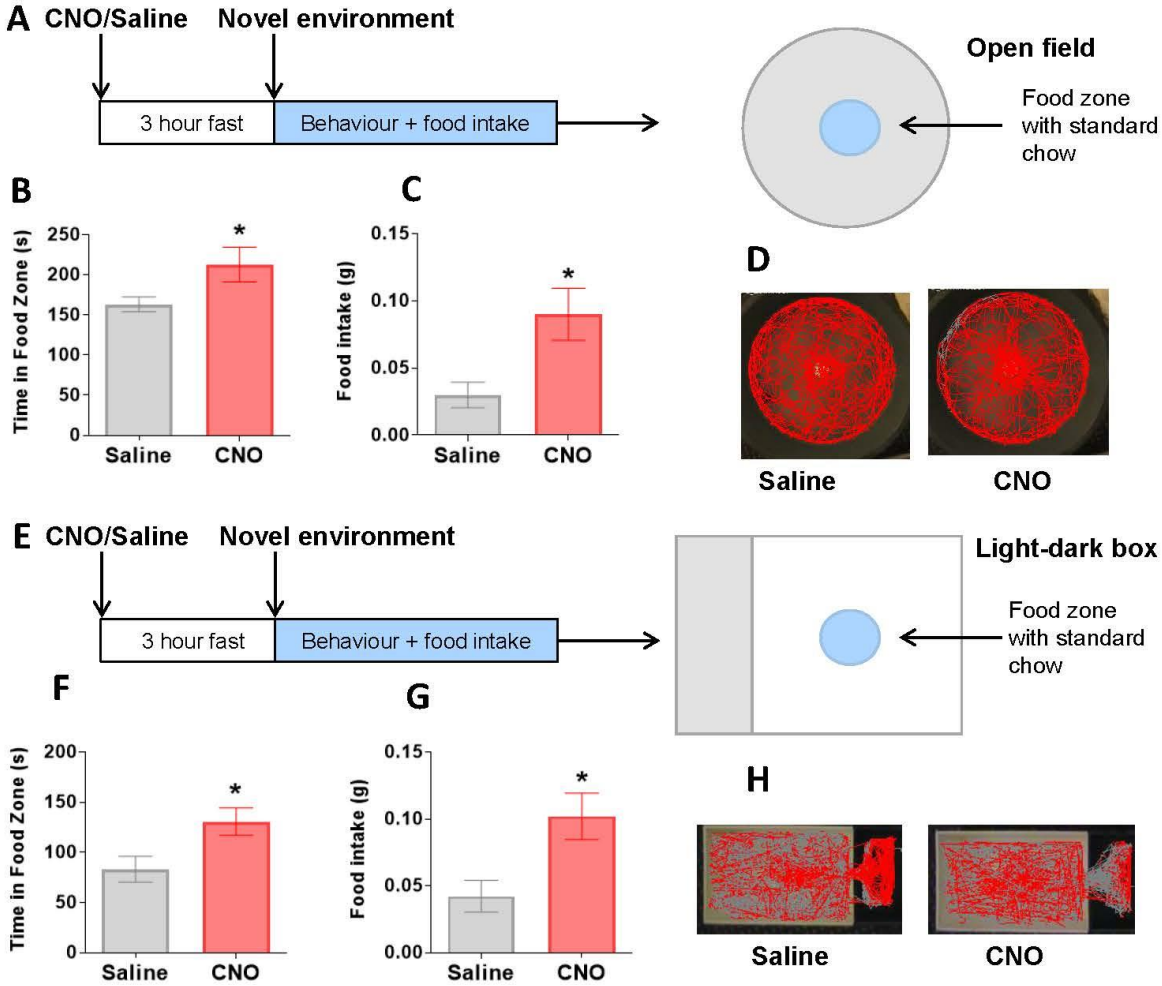


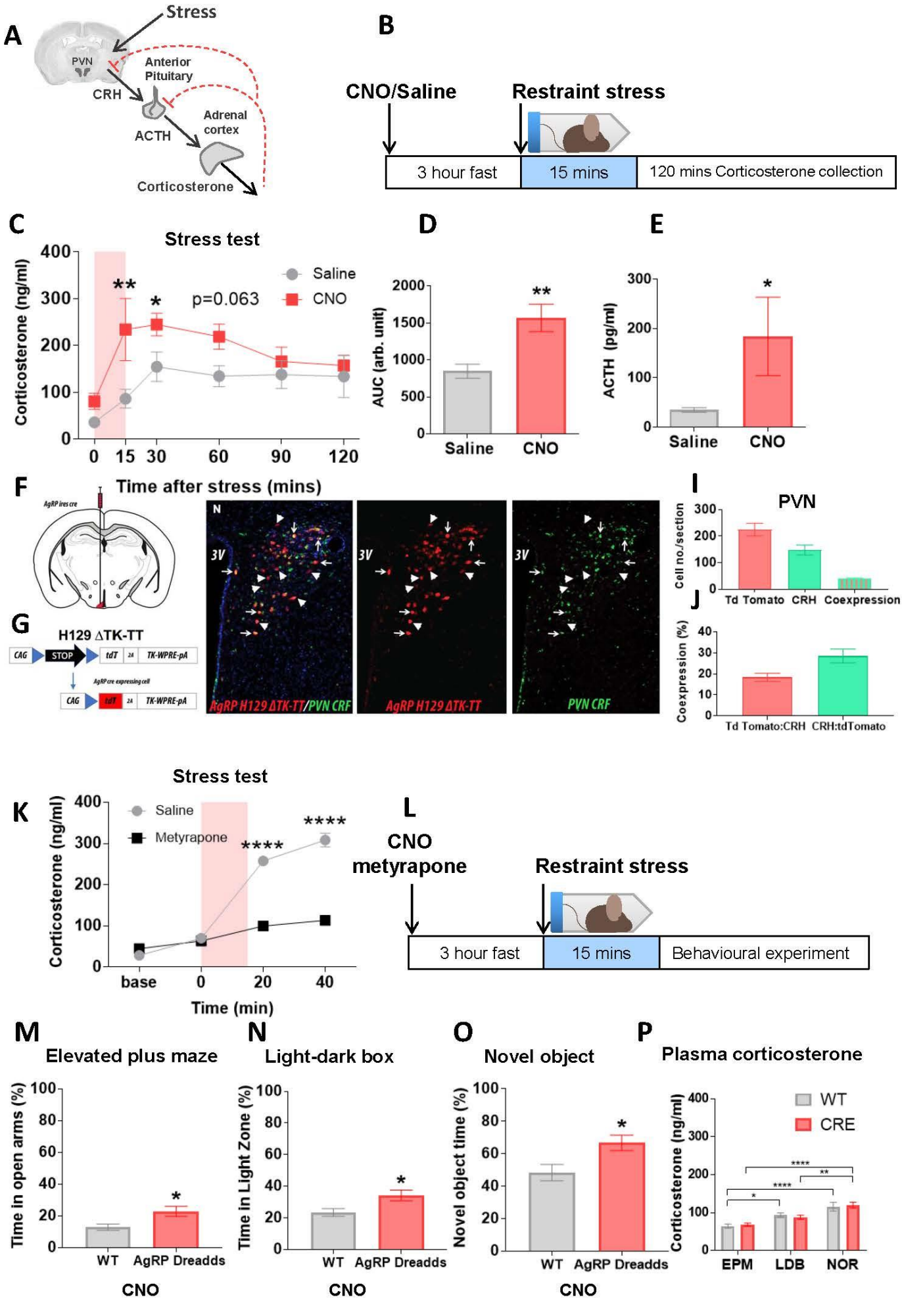
Figure 3 - Agrp activation promotes food-seeking and feeding behavior in a novel environment. Experiment approach for the open field as a novel environment (A). CNO increases time spent in the food zone and food intake after 30 minutes in the open field (B&C; n=11-12, Student's unpaired t-test). Representative track map from saline treated animal (left) and CNO treated animal (right) in open field (D). Experiment approach for the light-dark box as a novel environment (E). CNO increases time spent in the food zone and food intake after 30 minutes in the light-dark box novel environment (F&G; n=11-12, Student's unpaired t-test). Representative track map from saline treated animal (left) and CNO treated animal (right) in light-dark box (H). Data are expressed as mean ± sem and * indicates p<0.05.

Agrp activation promotes HPA axis response to acute restraint stress.

Previous studies demonstrate that fasting for 3 hours increases ACTH and plasma CORT levels (Dallman et al., 1999), suggesting that hunger sensing neurons interact and engage with the HPA axis. Given the behavioral changes observed following prolonged Agrp activation prior to an acute restraint stress, we then asked whether prior Agrp activation produced a differential effect on plasma CORT and ACTH levels in response to stress. CNO injection 3 hours before restraint stress produced a greater plasma CORT response to restraint stress with significant differences at 15 and 30 minutes after restraint stress, which was also reflected in a significant increase in AUC analysis (Fig 4C&D). CNO treatment 3 hours before restraint stress also significantly increased plasma ACTH 30 minutes post stress (Fig 4E). Next, we investigated whether Agrp neurons are synaptically connected to CRH neurons in the PVN using the cre-dependent trans-synaptic anterograde tracer H129 strain of HSV (H129 ΔTK-TT). 5 days after H129 ΔTK-TT injection into the ARC of Agrp-IRES-*cre* mice, tdTomato expression was observed in approximately 28% of CRH neurons of the PVN, indicative of a synaptically connected circuit arising from Agrp-cre neurons (Fig 4I&J). This reveals a potential neural circuit via which Agrp neurons can affect the HPA axis. Together these results suggest Agrp activation prior to an acute stressor primes the HPA axis and augments the hormonal response to stress.

Inhibition of CORT biosynthesis does not affect Agrp DREADD-induced behavioral changes.

Since Agrp activation prior to acute stress increased both CORT and adaptive behaviors, we then asked whether this relationship is simply an association or whether increased CORT acts to promote adaptive behaviors. To determine whether the observed increase in plasma CORT concentration after Agrp neuronal activation could be driving the behavioral changes in the elevated plus maze, light-dark box and novel object, we used metryapone (an inhibitor of CORT biosynthesis; 75mg/kg i.p.) and repeated the behavioral experiments shown in Figure 2, in a similar manner to that previously described (Füzesi et al., 2016). Metyrapone reduced plasma CORT concentrations following acute restraint stress compared to saline treated animals (Fig 4K). However, Agrp DREADD activated mice treated with metyrapone still spent significantly more time in the open arm of the elevated plus maze (Fig 4M), as well as the light zone of the light-dark box (Fig 4N), and significantly more time exploring the novel object in the novel object recognition task (Fig 4O). There was no difference in plasma CORT immediately following each behavioral test between Agrp DREADD expressing mice and WT controls, but CORT was greatest following the novel objection recognition task compared to the elevated plus maze for both groups (Fig 4P). These results indicate that an increase in plasma CORT is not required for Agrp-mediated adaptive behaviors following stress. Instead, it is likely the behavioral changes observed here are neurally mediated.


A
Stress
PVN
Anterior Pituitary
CRH
ACTH
Adrenal cortex
Corticosterone
B
CNO/Saline
Restraint stress
3 hour fast
15 mins
120 mins Corticosterone collection
C
Stress test
Corticosterone (ng/ml)
Saline
CNO
p=0.063
Time after stress (mins)
D
AUC (arb. unit)
Saline
CNO
E
ACTH (pg/ml)
Saline
CNO
F
AgRP ires cre
N
3V
AgRP H129 ΔTK-TT/PVN CRF
AgRP H129 ΔTK-TT
PVN CRF
G
H129 ΔTK-TT
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Coexpression (%)
Td Tomato:CRH
CRH:tdTomato
K
Stress test
Saline
Metyrapone
Corticosterone (ng/ml)
base
Time (min)
L
CNO
metyrapone
Restraint stress
3 hour fast
15 mins
Behavioural experiment
M
Elevated plus maze
Time in open arms (%)
WT
AgRP Dreadds
CNO
N
Light-dark box
Time in Light Zone (%)
WT
AgRP Dreadds
CNO
O
Novel object
Novel object time (%)
WT
AgRP Dreadds
CNO
P
Plasma corticosterone
Corticosterone (ng/ml)
WT
CRE
EPM
LDB
NOR

Figure 4 – Agrp activation promotes HPA axis response to acute stress but the HPA response does not mediate adaptive behavioral changes. Overview of the hypothalamic-pituitary-adrenal (HPA) axis (A). Experimental approach (B). Stress-induced plasma corticosterone when CNO was injected 3 hours before acute restraint stress (B; n=10, two-way repeated measures ANOVA followed by Sidak's multiple comparison test) with area under the curve (AUC) analysis (D; Student's unpaired t-test). Plasma ACTH 30 minutes after CNO or saline injection (E; n=6-9, student's unpaired t-test). Experimental approach showing unilateral injection of the cre-dependent anterograde H129 ΔTK-TT tracer in Agrp-ires-cre mice (F-H). Number of Td Tomato, CRH or co-expressing neurons in the PVN (I) and the percent coexpression (J); n=10. Injection of metyrapone prior to restraint stress reduces plasma corticosterone, compared to saline, during and after 15-minute restraint stress period (K; n=8, two-way repeated measures ANOVA followed by Sidak's unpaired t-test). Experimental approach using WT and Cre DREADD-expressing animals all treated with CNO and metyrapone (L). Treatment with Metyrapone does not attenuate differences in anxiety-like behavior observed following DREADD mediated activation of Agrp neurons in either the elevated plus maze (EPM) (M; n=5-10, Student's unpaired t-test) or light-dark box (LBD) (N; n=10-11, Student's unpaired t-test); nor does metryrapone treatment attenuate the increased novel object exploration time in the novel object recognition task (NOR) where the experimental design is equal to that shown in figure 2 (J) (O; n=11, Student's unpaired t-test). Plasma corticosterone did not differ between WT and Cre expressing animals however varied according to behavioral test (P; n= 10-11, two-way ANOVA with Sidak's multiple comparision test). Data are expressed as mean ± sem and * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$. Scale bars represent 60 µm.

Optogenetic activation of ARC Agrp neurons during acute restraint stress only is not sufficient for full expression of adaptive behaviors.

Previous studies demonstrate that prior activation of Agrp neurons enabled cued food-seeking behavior in a threatening environment (15). Our DREADD experiments support these findings and demonstrate that Agrp activation 3 hours prior to an acute stress promotes adaptive behaviors observed in Figure 2. In order to determine whether prolonged Agrp signaling prior to the onset of stress is required for adaptive behavioral responses, we sought to activate Agrp neurons solely during the restraint stress period. To achieve precise temporal control of Agrp neurons, a manipulation not possible with the use of DREADDs, we used a wireless optogenetic approach to activate Agrp neurons only during the restraint stress period before repeating behavioral experiments. We confirmed hChr2(H134R) expression by visualizing the expression of mCherry reporter in the ARC after all testing in all experimental animals. The mCherry reporter expression was confined to the ARC (Fig 5B, left) and co-expressed in NPY GFP neurons (Fig 5B, right), suggesting selectively targeted hChr2(H134R) expression in Agrp neurons. To functionally validate ChR2 expression in Agrp neurons, food intake was analysed in Agrp-IRES-*cre*::Npy GFP mice with hChR2(H134R) expression. Stimulation (20Hz, 3 sec on, 1 sec off) for 1 hour significantly increased food intake in the light phase compared to 1 hour no stimulation immediately prior, but was not different to 1 hour no stimulation post stimulation period (Fig 5C). In a real-time place preference paradigm, mice with Agrp photo-stimulation (20Hz, 3 sec on, 1 sec off) spent significantly less time in the stimulation-paired side of the apparatus compared to a baseline no stimulation period (Fig 5F), as previously reported (10). Agrp activation only during the restraint stress period significantly increased plasma CORT at 30 minutes post stress and AUC for 120 minutes post stress (Fig 5H&I). Agrp activation only during restraint stress increased time spent in the open arm of the elevated plus maze (Fig 5J) but had no significant effect on time spent in the light zone of the light dark box or time spent exploring the novel object (Fig 5K&L). Time spent in the open arms of the elevated plus maze

was significantly correlated with food intake (Fig 5M). Time spent in the light-zone of the light-dark box was not correlated with food intake (Fig 5N; p=0.0797). These results indicate that optogenetic Agrp activation timed with the onset of a 15-minute restraint stress period is not sufficient for the full expression of adaptive behaviors observed following prolonged Agrp DREADD activation occurring prior to the onset of acute stress.

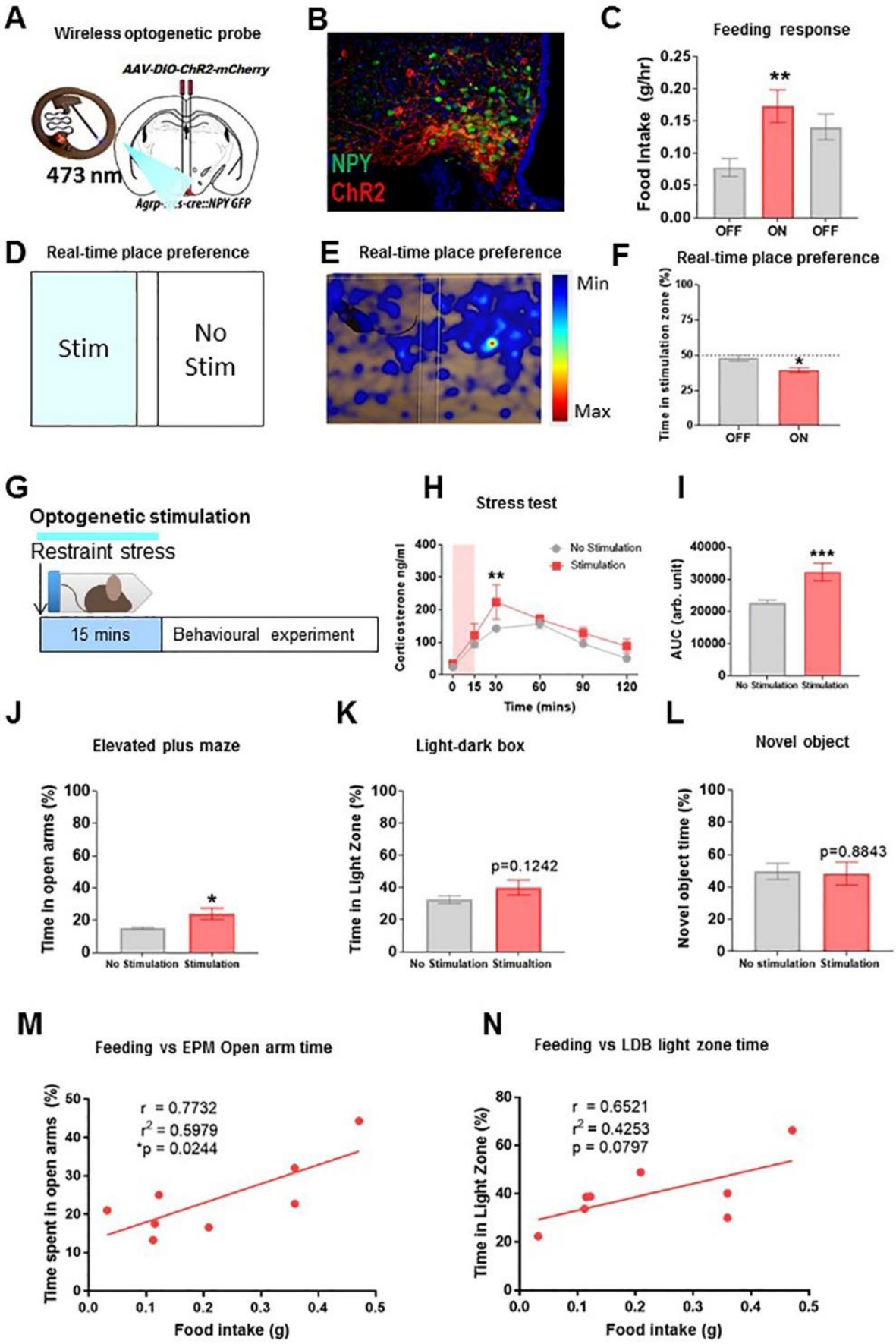


Figure 5- Optogenetic activation of Agrp neurons only during restraint stress period is not sufficient for full expression of adaptive behaviors. The cation channel ChR2, AAV-DIO-hChR2(H134R)-mCherry,

was injected bilaterally into the ARC nucleus of Agrp-ires-cre::NPY GFP mice (A). Representative image showing co-expression between NPY GFP and Agrp cre-dependent AAV-DIO-hChR2(H134R)-mCherry neurons (B). Food intake before, during and after 1 hour of optogenetic stimulation of Agrp neurons (20Hz, 3 seconds on, 1 second off) (C; n=23, one-way ANOVA with Tukey's post hoc test). Real-time place preference apparatus (D). Heat-map showing time spent in stimulation and no stimulation side of real-time place preference apparatus (E). Mice spent significantly less time on the stimulation-paired side of the real-time place preference apparatus during the stimulation period (F; n=11, Student's paired t-test). Experimental approach for optogenetic experiments (G). Mice with Agrp activation only during restraint stress period had significantly increased plasma corticosterone at 30 minutes after the onset of restraint stress (H; n=8-14, two-way ANOVA with Sidak's multiple comparison test) and significantly greater AUC for corticosterone over the entire 120-minute period (I; n=8-14, Student's unpaired t-test). Mice with Argp activation only during restraint stress spent significantly more time in the open arms of the elevated plus maze (J; n=8-14, Student's unpaired t-test) but did not differ from control mice for behavior in the light-dark box (K) and novel object recognition task (L). Time in open arms of elevated plus maze was significantly correlated with food intake (M; n=8, Pearson's correlation) but time in light-dark box vs food intake did not reach significance (N; n=8, Pearson's correlation).

2.5 Discussion

In this study, we show that Agrp activation prior to the onset of an acute stressor coordinates a diverse range of adaptive behaviors that prioritise food seeking and food intake. Agrp activation reduced anxiety-like behavior, increased locomotor activity and improved memory recall in the face of an acute stressor, while simultaneously potentiating the HPA axis response to stress. Unexpectedly, inhibiting the biosynthesis of CORT had no effect on the Agrp DREADD-mediated expression of adaptive behaviors immediately following an acute stressor. Our results are in line with prior studies that show activation of Agrp neurons reduces anxiety in the face of threats (Padilla et al., 2016), promotes conditioned food seeking in a threatening environment (Jikomes et al., 2016) and increases novelty-seeking behavior (Dietrich et al., 2012, 2015). However, this is the first study to demonstrate Agrp neuronal activation promotes the HPA axis response to an acute stressor, as well the first study to show that Agrp neuronal activation improves memory recall in a novel object recognition task. These results highlight that Agrp neurons are a key neuronal population through which fasting both increases CORT levels (Dallman et al., 1999), and promotes memory recall (Towers et al., 2017).

Although Agrp activation potentiates plasma CORT release in response to stress at the time when behavioral experiments were conducted, our studies with metyrapone unequivocally demonstrate this increase in plasma CORT did not affect the expression of the behavioral changes observed. Recently, Kim et al elegantly described that CORT is not involved in the active suppression of CRH neuronal responses to acute stressors but rather plays an important role to inhibit tonic CRH neuronal activity in the absence of stressors (Kim et al., 2019). It has recently become clear that stress elicits two distinct neuronal responses; plasticity driven by endocrine feedback on stress hormone receptors and plasticity controlled by stress-induced neural activity (Bains et al., 2015; Tasker & Herman, 2011). Many recent studies highlight that CRH neural activity promotes adaptive coping behaviors immediately after stress (Füzesi et al., 2016) and encodes reward valence (Füzesi et al., 2016; Kim et al., 2019; Yuan et al., 2019), and that these normal neural and behavioural responses need to occur unopposed by any feedback effects of CORT. Our results suggest that Agrp activation potentiates this downstream stress-evoked neural network, which includes CRH neurons independent of plasma CORT feedback, ultimately resulting in a resilient state in which anxiety is suppressed and salience is given to tasks related to finding and consuming food, despite a full capacity to mount a stress response. This statement is strengthened by our cre-dependent anterograde tracing experiments using the HSV variant H129 ΔTK-TT showing that Agrp neurons are synaptically connected to PVN CRH neurons. However, since the H129 ΔTK-TT virus is trans-synaptic we cannot determine whether this is a direct or indirect connection, although the available evidence would suggest this is indirect since light activation of Agrp neurons failed to evoke inhibitory post-synaptic currents in 9/9 PVN CRH neurons (Garfield et al., 2015).

The nature of a stress-evoked network downstream of Agrp neurons remains unknown, however given that fast acting actions of Agrp neurons are mediated by NPY and GABA (Krashes et al., 2013; Ruud et al., 2020)we assume this involves inhibition of downstream targets. Potential targets include the central or medial

amygdala (CeA, MeA) given that Agrp neurons project to the CeA and MeA (Betley et al., 2013; Padilla et al., 2016). The Agrp to CeA circuit appears not to affect feeding behaviour (Betley et al., 2013), suggesting a role for this circuit outside of simply food consumption; while Agrp to MeA projections reduce territorial aggression, an effect mediated by MeA^{NPY1R} neurons, and moderately increase food intake, though not to the same level as Agrp to PVN projections (Padilla et al., 2016). In addition, inhibiting CRH neurons in the CeA resulted in reduced anxiety-like behavior and improved novel object recognition, while activating these neurons had the opposite effect (Paretkar & Dimitrov, 2018). Taken together, these studies suggest that the behavioral changes occurring after Agrp activation may involve acute neurally-mediated inhibition of downstream amygdala targets, although this needs to be experimentally tested.

It is important to note that previous studies have identified protective roles for CORT to restrict anxiety (Rao et al., 2012), although over different time frames that are not consistent with the studies herein. For example, increased CORT delivered in drinking water for 12 hours prior to acute stress, allowed sufficient time to produce observable changes in spinogenesis in basolateral amygdala medium pyramidal neurons, which subsequently restricted anxiety days later when exposed to acute stressors. In our study, Agrp neuronal activation only increased plasma CORT concentrations 15-30 mins after acute stress onset, precluding the increase in CORT influencing acute anxiolytic behavior measured immediately after acute restraint stress.
Although plasma CORT feedback unequivocally did not influence the anxiolytic effect of Agrp neuronal activation on acute stress, our studies show that chronic or acute Agrp activation with DREADDs or ChR2 respectively, increases plasma CORT after acute stress. The role of this Agrp-induced CORT response to stress remains unknown, although it has been recently shown that CORT drives Agrp activation and is crucial to stimulate food intake under conditions of fasting and hypoglycaemia (Perry et al., 2019). CORT increases Agrp firing rates but has no effect on non-Agrp ARC neurons in the absence of synaptic transmission (Perry et al., 2019). In addition, CORT produces increased Agrp activity at least up to 60 mins after injection as measured by fibre photometry and inactivating CORT actions in Agrp neurons by over-expressing 11b-hydroxysteriod dehydrogenase 2, a glucocorticoid inactivating enzyme, restricts feeding after fasting, during hypoglycemia and hypercorticosteronemia (Perry et al., 2019). These observations are consistent with previous studies linking CORT with weight gain, hyperphagia and orexigenic gene expression (Akabayashi et al., 1994; Lu et al., 2002; Ponsalle et al., 1992; Shibata et al., 2016) although Agrp does not appear to be responsible for most of the glucocorticoid-induced adverse metabolic effects, as similar effects are seen in Agrp wt and ko mice (Sefton et al., 2019). It is possible therefore that Agrp-induced CORT might function to recover energy lost to the acute stress. This idea is supported by our observations that plasma CORT returns to similar levels seen in control mice 2-hours after the onset of acute stress.

Agrp activation *prior* to the onset of restraint stress appears to be important in the expression of the full range of assessed adaptive behaviors, as optogenetic activation of Agrp neurons during the restraint stress period, only results in an anxiolytic response in the elevated plus maze but not light-dark box and had no effect on memory recall in the novel object recognition task. Our data are supported by those of Jikomes et al. in which pre-emptive Agrp neural activation before being placed a threatening cued foot shock context (psychological

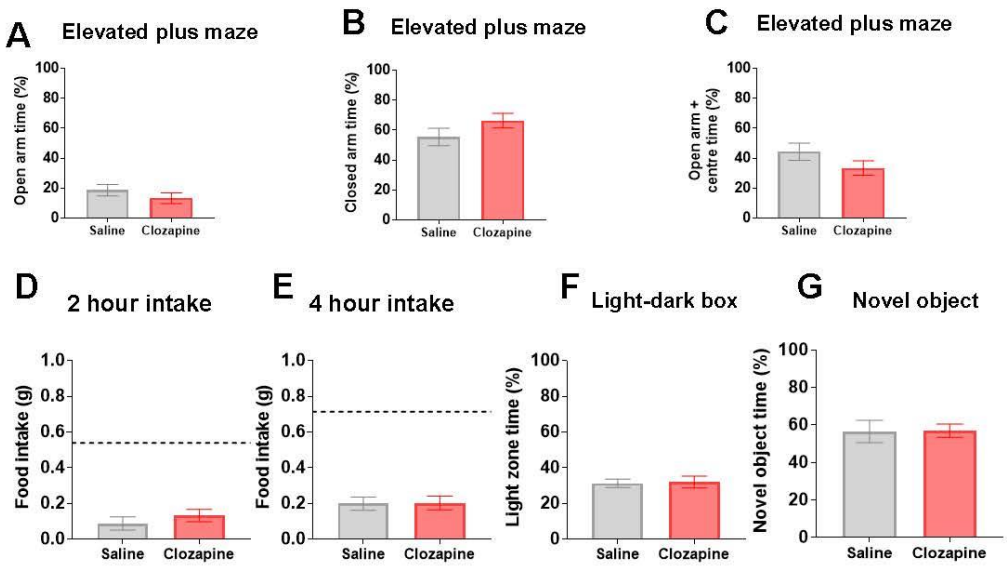
stress) facilitated food seeking, whereas activation of Agrp neurons once already in the threatening environment did not (Jikomes et al., 2016). Interestingly, acute optogenetic Agrp activation only produced an anxiolytic response in the elevated plus maze but not light dark box and Agrp-induced food intake correlated significantly with time spent in elevated plus maze open arms. This suggests that entry into the light zone from the relatively safe dark zone produces a stronger anxiogenic response compared to the elevated plus maze, an observation that is confirmed by plasma CORT in our studies (Fig 4P). In terms of memory recall, only activation of Agrp neurons with DREADDs prior to acute stress increased memory recall in a novel object recall task. We did not observe any difference in memory recall in the novel object recognition task when Agrp activation was restricted to the restraint stress period. It is known that stress-induced anxiety reduces novel object recognition (Cazakoff et al., 2010), explaining why control mice in our novel object recognition experiments failed to recognise the novel object. At the end of behavioral tests, we observed that plasma CORT was highest in novel object recognition >light dark box> elevated plus maze, supporting the idea that our novel object recognition paradigm was more stressful than other tests. This also offers an insight into why activation of Agrp prior to, but not during, acute stress improved memory recall, since activation of Agrp neurons prior to acute stress produced a greater anxiolytic effect compared to Agrp activation during acute restraint stress. Previous studies show that Agrp activation underlies learning and decision making related to food-seeking in environments previously paired with food availability (Burnett et al., 2016). Our results demonstrate that Agrp neuronal activation prior to acute stress helps memory formation not just in the context of learned associations with food availability but also with objects unrelated to food consumption.

Our results reinforce the notion that hunger, signalled via Agrp neurons, elicits a complex set of behaviors not simply limited to food consummatory behavior. These adaptive behavioral responses such as the suppression of anxiety and increased memory recall in response to acute stress as shown herein, help an organism effectively focus on relevant salient tasks, such as food seeking. Presumably during hunger or starvation (prolonged Agrp activation) when all Agrp neural circuits are activated, hunger related information is relayed to regions that promote food intake but also those regions engage adaptive behavioral responses, such as reduced anxiety and memory recall. Under these conditions, the degree of Agrp activity (current hunger state) is expected to influence the magnitude of the adaptive behavioral response, which may explain why Agrp activation prior but not during acute restraint stress is required for the full expression anxiolytic and memory behaviors. Indeed, this is reinforced by the observation that feeding in response to photostimulation significantly correlates with time spent in elevated plus maze open arms after photostimulation during restraint stress only.

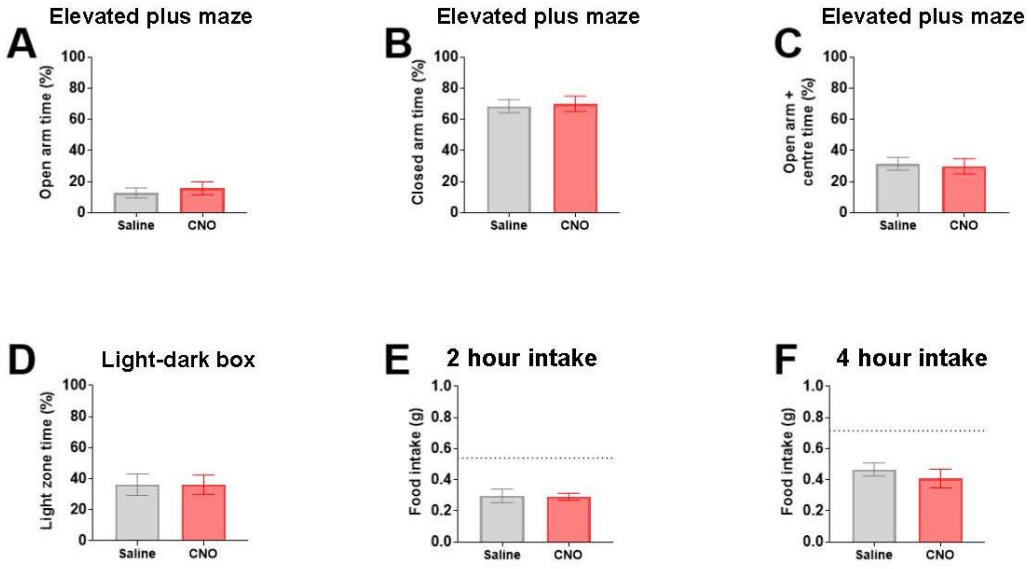
Based on our data, we postulate that Agrp hunger-sensing pathways would promote food-seeking behaviors while simultaneously suppressing anxiety and enhancing memory formation in acutely stressful situations. This is particularly relevant for prey species in which hunger increases the need to seek and forage for food in unfamiliar environments but also enables an organism to deal with imminent threats and stressors in this unfamiliar environment. From an ethological standpoint, the ability to remain alert and focused, while

suppressing anxiety and promoting memory recall, is considered optimal for current and future food-seeking opportunities in a stressful or potentially dangerous environment.

2.6 Supplemental figures



Supplemental Figure 1 – CNO (1mg/kg) does not influence anxiety-like behavior or food intake in C57/BL6 mice without DREADD expression. No difference between saline or CNO treatment in the percentage of time spent in the open arm, closed arm or open arm and center combined (A-C; n=8). No difference in the percentage of time spent in the light zone of the light-dark box (D; n=8). No difference in 2-hour or 4-hour food intake under non-stressed conditions, where the experimental design is equal to fig 3B but without stress. Lines indicate Agrp DREADD-induced food intake from experiment shown in figure 2C-D (E,F; n=8). Data are expressed as mean ± sem.



Supplemental Figure 2 - Clozapine does not influence anxiety-like behavior or food intake in C57/BL6 mice without DREADD expression. No difference between saline or clozapine treatment in the percentage of time spent in the open arm, closed arm or open arm and center combined (A-C; n=8). No difference in 2-hour or 4-hour food intake under non-stressed conditions, where the experimental design is equal to figure 2B but without stress. Lines indicate Agrp DREADD-induced food intake from experiment shown in figure 2C-D (D,E; n=8). No difference in the percentage of time spent in the light zone of the light-dark box (F; n=8). No difference in novel object interaction time (G; n=7-8). Data are expressed as mean ± sem.

3. Chapter Three: mPFC inputs into the LH suppress feeding and motivated reward-seeking

3.1 Introduction

Over the past 20 years, studies examining the neural regulation of food intake have primarily focused on homeostatic control systems, usually located within the hypothalamus and brainstem. Agouti-related peptide (Agrp) and pro-opiomelanocortin (POMC) expressing neurons of the arcuate nucleus of the hypothalamus are examples of two extremely important homeostatic neural populations that play an incontrovertible role in food intake. For example, genetic ablation of Agrp neurons in adulthood leads to anorexia (Luquet et al., 2005), whereas genetic deletion of the melanocortin system (POMC neurons, peptide processing enzymes or melanocortin receptors) leads to severe obesity (Coll et al., 2004). In fact, genetic mutations in various components of the melanocortin system are the most common forms of human heritable obesity (Boutin & Froguel, 2001). However, melanocortin mutations are not overly common within society and account for a small proportion of global obesity rates (1-4%) (Boutin & Froguel, 2001).

A primary cause of obesity in humans is the overconsumption of calories, particularly from highly palatable and energy dense foods (*World Health Organisation*, 2000). High energy foods are innately rewarding to animals and humans, ensuring they are motivated to seek and consume these foods. While this originated as an adaption to promote survival in environments where food is scarce, the rewarding nature of high energy foods in the modern obesogenic environment now poses a challenge to the maintenance of a healthy bodyweight. Indeed, the overconsumption of food in humans is largely driven by non-homeostatic factors, such as environmental and cognitive influences and therefore there is a greater need to understand non-homeostatic neural systems that influence food intake. This is especially important considering efforts to reduce overweight and obesity through lifestyle measures such as diet and exercise generally have limited efficacy, with many overweight and obese individuals reporting they are unable to adhere to dietary changes (Puhl et al., 2008). Moreover, evidence from human genetic studies show that obesity is largely defined a heritable disease affecting the neural control of overeating (O'Rahilly & Farooqi, 2008). In order to understand the influence of non-homeostatic feeding pathways on the neural control of feeding in humans, we have worked in collaboration with researchers at Monash University to identify neural pathways in human imaging studies.

Neuroimaging studies in humans have implicated the medial pre-frontal cortex (mPFC) in processing of food cues (Schur et al., 2009). A range of functions have been ascribed to this brain region including decision making, valuation of stimuli and inhibitory control (Carlén, 2017; Delgado et al., 2016). In addition, the mPFC appears to be a key brain region that is involved in aspects of human addiction, such as regulation of reward systems and higher-order executive control (Goldstein & Volkow, 2011). Indeed, the mPFC is a brain region frequently identified as differentially active in neuroimaging studies in human obesity. Obese individuals consistently display stronger activation of the mPFC in response to images of food than healthy weight

controls (Brooks et al., 2013; Kennedy & Dimitropoulos, 2014; Stoeckel et al., 2008), suggesting they may be more sensitive to food cues. In addition to this, when choosing between available unhealthy and healthy food options (not images) in a fasted state, obese individuals have stronger activation of the mPFC coupled with weaker activation of the hypothalamus, the brain region that controls energy homeostasis (Harding et al., 2018).Together human neuroimaging data suggest that obese individuals have greater activity in brain regions associated with reward valuation and weaker activity in regions that regulate energy intake. This pattern of brain activity allows for a scenario where food consumption is primarily driven by the non-homeostatic hedonic properties of a food, rather than internal signals of hunger and satiety.

While human neuroimaging studies are useful in highlighting brain regions implicated in obesity, they have a number of limitations. Firstly, these studies use functional magnetic imaging (fMRI) to detect blood oxygen levels and infer differences in activity and therefore do not indicate the types of neurons that might be active or the neurotransmitters or neuropeptides involved in a particular response. In addition, the human hypothalamus is relatively small and difficult to image, limiting what can be concluded about this region (Huerta et al., 2014). From animal studies, we know that the hypothalamus is a functionally and anatomically heterogeneous region essential for regulating energy balance. However, only few human imaging studies have considered analysing sub regions of the hypothalamus (broadly medial and lateral) and how these are functionally connected to the cortex (Contreras-Rodríguez et al., 2017; Kullmann et al., 2014) . It is important to note that functional connectivity assessed through fMRI does not directly represent anatomical connectivity as a number of regions that are not connected still observe functional correlations (Buckner et al., 2013). However, unpublished work from our collaborators at Monash University, using spectral dynamic causal modelling – which is able to predict the direction of communications between brain regions – suggests that the mPFC projects directly to the hypothalamus and activity of this circuit appears to be implicated in obesity (Voight et al 2020, manuscript submitted: *Hunger state and adiposity influence causal interactions between neural systems for feeding, reward and choice*). Studies in rodents support the existence of this circuit with clear monosynaptic projections reported from the mPFC to the LH (Gabbott et al., 2005; Hahn & Swanson, 2010; Vertes, 2004). While the mPFC projections to the basolateral amygdala (BLA) and nucleus accumbens (NAc) have been thoroughly investigated, with dopamine 1 receptor (D1) expressing mPFC to BLA projections reported to induce feeding (Land et al., 2014), very little is understood about the role of mPFC neurons that project to the LH.

Classical electrostimulation and lesion studies in rodents have defined the lateral hypothalamus (LH) as a key region of both feeding and reward related behaviours, while by contrast the medial hypothalamus is associated with satiety (Anand & Brobeck, 1951; Hoebel & Teitelbaum, 1962; Margules & Olds, 1962; Olds & Milner, 1954). Since then, these early studies of the LH have been confirmed using optogenetics. Acute photoactivation of GABAergic LH neurons (*Vgat*-expressing neurons; LH^{Vgat}) rapidly induces voracious feeding, and promotes self-stimulation (Jennings et al., 2015) mirroring the results of earlier studies using crude electrical self-stimulation to the LH area (Olds & Milner, 1954). By contrast, photoactivation of glutamatergic (*Vglut2* expressing neurons) within the LH (LH^{Vglut2}) suppresses feeding and induces aversion

(Jennings et al., 2013). The current understanding is that LH^{Vgat} neurons and LH^{Vglut2} neurons have bidirectional effects on feeding.

One unique feature of the LH is its substantial connectivity to a diverse range of brain regions (Barone et al., 1981; Betley et al., 2013; Gabbott et al., 2005; Kita & Oomura, 1982; Yoshida et al., 2006). The LH receives a number of inputs from both hypothalamic and extra-hypothalamic regions, and as a result is thought to be a key integrator of metabolic signals and environmental information – such as food cues (Clarke et al., 2018). While the inputs from limbic regions have been well characterised (Carus-Cadavieco et al., 2017; Jennings et al., 2013; O'Connor et al., 2015), less is known about the cortical inputs to the LH. The rodent LH receives inputs from a number of cortical regions that appear to be implicated in human obesity including the mPFC (as described above) and the insular cortex (Gabbott et al., 2005; Hahn & Swanson, 2010; Kita & Oomura, 1982; Vertes, 2004; Yoshida et al., 2006).

Recently, a study by Wu et al (2020) reported that activation of monosynaptic glutamatergic inputs from the right anterior insula cortex to the LH suppress feeding in fasted mice and induce aversion (Wu et al., 2020). Also, this study reported the right anterior insula cortex punitive projection neurons are activated in response to visceral aversive stimuli, suggesting this circuit acts to override metabolic signals of hunger when an organism is receiving interoceptive signals of nausea or inflammation (Wu et al., 2020). Interestingly, human imaging studies report that the insula cortex, like the mPFC, has greater blood flow, and hence activity in patients with obesity when viewing images of high calorie food (Huerta et al., 2014; Schur et al., 2009). However, both the mPFC and insula cortex regions also appear to be hyperactive in patients with anorexia when exposed to food cues (Ellison et al., 1998b; Uher et al., 2004). Due to the limitations of fMRI studies, it is not possible to determine whether increases in blood flow indicate increased activity of interneurons in these regions, and therefore an inhibition of projection neurons, or an increase in activity of projection neurons themselves. Given that both obesity and anorexia involve the overriding of homeostatic signals it is possible that perturbations to activity in these cortical regions is an underlying factor contributing to these conditions.
The mPFC-LH circuit has not yet been directly investigated in the context of feeding and reward. Biro et al (2018) report that photoactivation of the glutamatergic mPFC terminals projecting to the LH increases aggressive behaviours (Biro et al., 2018), however this study did not investigate any effects of circuit activation on food intake or motivational valence. Photoactivation of the subpopulation of dopamine receptor 1 (D1) expressing neurons of the mPFC has been demonstrated to increase feeding, and this appears to be driven through projections to the basolateral amygdala (Land et al., 2014). While sparse fibres from mPFC D1 neurons were reported to be present in the LH in this study, the effect of activation of this circuit was not considered (Land et al., 2014). Supporting a role for the mPFC-LH circuit in control of feeding behaviour, μ-opioid receptor agonism of mPFC neurons results in feeding and hyperactivity, and these neurons appear to project indirectly to orexin expressing neurons in the LH (Mena et al., 2013).

Given human imaging data implicates the mPFC to LH circuit in obesity and response to external feeding cues, and that animal studies report that both the mPFC and LH regions can influence feeding behaviour, it

is reasonable to hypothesise that the mPFC-LH circuit has a role in linking non-homeostatic and homeostatic drives to eat. In this study, we sought to determine the effect of manipulating the mPFC-LH circuit in mice. We hypothesise, based on studies investigating cortical inputs to the LH and studies of the LH more broadly, that this circuit exerts control over feeding and reward seeking behaviours.

3.2 Methods

Animals

All experiments were conducted in accordance with the Monash Animal Ethics Committee guidelines. C57BL/6 male mice were obtained from the Monash Animal Services facility. Vglut1-ires-cre male mice on a C57BL/6 background were obtained from the Jackson Laboratory (B6.Slc17a7-IRES2-Cre-D; stock number 023527) and bred in the Monash Animal Services Facility. Mice were aged between 8-10 weeks at beginning of experiments. Mice were maintained on a 12-hour light-dark cycle with *ad libitum* access to standard chow (Rat and mouse pellets, Specialty Feeds, Western Australia) and water under standard laboratory conditions (21°C). In the mice that underwent calorie restriction, mice were maintained at 85% of their original body weight before experiments began. All mice were group housed following surgery and throughout the duration of behaviour experiments. Mice were individually housed for food intake experiments, feeding cage experiments and operant conditioning experiments. Caspase and optogenetics experiments were conducted within the light phase (7am-7pm). DREADDs experiments were conducted through the dark phase with mice on a reverse light cycle (11pm-11am). Mice were handled for 5 min each day for five consecutive days leading up to behaviour experiments.

Viruses and Surgical procedures

Mice were anesthetised using isoflurane (5% for induction, 2% for maintenance) and positioned on a stereotaxic frame (Stoelting). A Neuros syringe (Hamilton, Reno, NV, USA) was used to deliver virus to the target regions; LH (-1.4mm Bregma; +/- 1.0mm lateral; -4.8mm ventral from surface of brain) and mPFC (+1.5mm Bregma; +/- 0.3mm lateral; -1.8mm ventral from surface of brain). For viral tracing experiments 100nl of CTb-Alexafluor594 (Abcam) or pAAV-Syn-ChR2(H134R)-GFP (AAV Retrograde; Addgene) was injected bilaterally into the LH. To chronically ablate the mPFC to LH circuit CAV2-cre-GFP (Montpeiler Vector Core, France) or AAV-pgk-Cre (retrograde; Addgene) was injected bilaterally into the LH at a volume of 200nl and the cre-dependent caspase virus (AAV-flex-taCasp3-TEVp; UNC vector core, gifted by Nirao Shah & Jim Wells or AAV-GFP; Addgene for controls) was injected bilaterally into the mPFC at a volume of 300nl. To insert the stimulatory DREADDs into the mPFC to LH projection neurons AAV-pgk-cre (retrograde; Addgene) was injected bilaterally into the LH at a volume of 100nl before the cre-dependent viral construct encoding for the stimulatory DREADDs receptor (pAAV5-hSyn-DIO-hM3D(Gq)-mCherry; Addgene or AAV-GFP; addgene for controls) was injected bilaterally into the mPFC at a volume of 300nl. For optogenetics experiments a retrograde dependent viral construct was injected (AAV-DJ EF1a-DIO-hChR2(H134R)-mCherry; Addgene) bilaterally into the LH of Vglut1-cre mice and WT littermates at a volume of 100nl. A wireless antenna (Fig 5A; 9.8mm diameter, 1.3mm thickness, 6mm probe length; 30 mg weight; Neurolux) was then implanted above the mPFC unilaterally and fixed to the skull of the mouse using the procedure described in Shin *et al* 2015 (Shin et al., 2017).

Immunohistochemistry

After experiments, mice were deeply anesthetised and then transcardially perfused with 0.05M phosphate

buffered (PB) saline followed by 4% *w/v* paraformaldehyde (PFA) in 0.1 M PB. Brains were post-fixed in 4% PFA in 0.1 M PB overnight before being transferred to 30% sucrose solutions. Coronal sections of the whole brain were cut at 30 μm in sets of four. A one in four series of tissue sections was washed in 0.1 M PB three times before being blocked in 1% H_2O_2 in 0.1 M PB for 15 min and then washed again. Tissue was then placed in 0.3% Triton X-100 in 0.1 M PB and 4% normal horse serum for one hour before an overnight incubation at 4°C in primary antibody: anti-NeuN (rabbit, 1:1000, Abcam), anti-mcherry (chicken, 1:1000, Abcam). Tissue sections were then washed again before a 90 min incubation in secondary antibody at room temperature; goat anti-rabbit IgG AlexaFluor 488 (1:400; Life Technologies), goat anti-chicken IgG AlexaFlour 594 (1:400, Life Technologies), Alexa fluor goat-antirabbit IgG (1:400, Life technologies). The sections were then mounted and coverslipped with hard set mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) (Vector) used to counterstain DNA. To confirm cell ablation in caspase experiments images of NeuN stained tissue were analysed using Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.) to determine % area of cell coverage.

Optogenetic stimulation

For all optogenetic experiments light stimulation was delivered at 20Hz, 3 seconds on 1 second off using wireless LED probes (Neurolux) as described under surgical procedures above. An electromagnetic field was constructed around an animal cage (440mm X 340mm with wall height 200mm) and connected to a power distribution control box (12Amps; 10W; Neurolux) which delivered radio frequency power to the wireless LED probes. Prior to each experiment the antenna was tuned to the power distribution control box to ensure the impedance of the power source and antenna matched. Each antenna was tuned to ensure a standing wave ratio of <1.3 and an impedance of 50Ω. Blue light delivery (470nm) was in the range of 1-5mW as calculated using the irradiance calculator provided by Neurolux (Fig 9C).

DREADDs experiments

For activation of DREADDs mice were injected in the intraperitoneal cavity with CNO (1mg/kg; Sigma) 30 minutes to 3 hours before each experiment commenced.

Behaviour

Behavioural analysis

All behavioural analysis was performed blinded to treatment using (Ethovision version 14.0.1322, Noldus Information Technology).

Elevated plus maze

The elevated plus maze (400 mm above floor) consisted of two open arms (310 mm X 50 mm) and two closed arms (310 mm X 50 mm with 150 mm high barriers). Mice were placed in the centre of the plus maze facing

an open arm and allowed to explore the apparatus for the duration of the test (6 minutes). The time spent exploring both open arms was measured. The time spent in the centre of the plus maze was excluded.

Light dark box

The light-dark box (285 mm wall height) was constructed from wood and consisted of a larger light chamber (480 mm X 295 mm; painted white) and a smaller dark chamber (150 mm X 295 mm; painted black) separated by a small opening. Mice were placed in the centre of the dark chamber and allowed to move freely about the two chambers for the duration of the test (6 minutes). The time the mouse spent in the light chamber was measured.

Open field

Mice were placed in the outer ring of a large open field (800 mm diameter with wall height 300 mm) and allowed to freely move about the apparatus for the duration of the test (6 minutes). Time spent in the centre zone (200 mm diameter) was measured.

Baited behavioural tests

Baited behavioural tests have previously been optimised in our lab (Lockie et al., 2017). Mice were exposed to peanut butter chips (Reeces, USA) randomly three times during the week leading up to the baited behavioural tests. Mice either had *ad libitum* access to chow, or were fasted overnight (15 hours; for caspase experiments) or 3 hours before the onset of the dark phase (6 hours in total; DREADDs experiments). One peanut butter chip was weighed and then placed in the centre of the light chamber of the light-dark box described above. Mice were placed in the dark zone and then given six minutes to freely explore the apparatus. The time spent in the food zone (50mm radius around the pellet), the amount of the pellet consumed, entries into the food zone and entries into the dark zone were measured. On a separate day mice were placed into the open field, which was baited with three peanut butter chips, placed in the centre region. Mice were placed in the outer ring of the open field and then given six minutes to move about the apparatus. The time spent in the food zones (50mm radius around pellets), amount of pellet consumed, entries into food zone and entries into the outer zone were measured. All mice completed each baited test in both the fed and fasted states separated by one week. Groups were counter balanced to the order of treatment.

Real-time place preference

Mice were given 10 minutes to explore the real-time place preference apparatus (385mm X 245mm with wall height 170mm) before 20 minutes of stimulation (20Hz, 3 seconds on, 1 second off) only one side of the apparatus (Fig 5D). The box was wired so that sufficient power to light the LED would only be delivered to one half of the box (stimulation side). Alfoil was used to cancel the electromagnetic field on the no stimulation side of the box, however low levels of light were still observed at the very edge of the stimulation side so the centre zone was labelled as a neutral zone and not included in the analysis. Preference for stimulation period was calculated before and during stimulation based on time spent in each zone.

Operant conditioning

Feeding experimental devices (FED; version 3.0; openbehaviour.com, USA) were placed in the home cage with a divider attached to prevent mice from climbing the device (Nguyen et al., 2016). FEDs were filled with sucrose pellets as rewards (Test Diet Sucrose tablet 20 mg, Richmond, IN, USA). In both DREADDs and optogenetics experiments mice were trained for 7-10 days beginning at a fixed ratio (FR) of one for two hours for 3 days, then to FR 3 for two hours for two days, then to FR 5 for two hours for two days or until they reached a correct response rate of 75% or above (Figure 8A). For DREADDs experiments, a progressive ratio task (PR; where nose pokes required to obtain a sucrose pellet increase exponentially) was performed in fed and fasted (6 hours) states in the dark phase for 5 hours, separated by a week. Active and inactive pokes, pellets acquired and breakpoint – the number of nose pokes at which responding ceases, were recorded throughout each session. Mice were counter balanced to the order of treatment. In the week between PR sessions mice were trained on two days at FR 5 to ensure their response rate was maintained above 75%. For optogenetics experiments a PR was performed under conditions of no photostimulation and photostimulation, and again mice were counterbalanced to order of treatment. Each PR session lasted 5 hours and mice had ad lib access to chow during this period. Between each session mice were maintained for two days at FR 5 to ensure their response rate was maintained above 75%.

For extinction, FEDs were placed in the home cage for two hours each day over the 15-day period. During this period mice can freely nose poke however the light and sound stimuli are no longer paired with the active side and no pellets are delivered.

Food and liquid intake

Home cage food intake

For DREADDs and caspase fasting re-feeding during the light phase food was removed two hours before the onset of the dark phase and returned two hours after the beginning of the light phase (Fig 6I). For optogenetics fasting re-feeding experiments food was removed 1 hour before the onset of the dark phase and returned 1 hour after the beginning of the light phase (Fig 9D). For DREADDs experiments mice were injected with CNO (1mg/kg; Sigma) 1 hour before food was returned and food intake was measured at two hours and 4 hours (Fig 6I). For optogenetics experiments mice were transferred to a large wired cage and their home cages were placed inside. Photostimulation was delivered over a 2-hour period while food intake was measured hourly (Fig 9D). For feeding during the dark phase food, was removed one hour before dark phase onset and returned 3 hours later, CNO (1mg/kg; Sigma) was injected 1 hour before food was returned and intake was measured at two hours and 4 hours (Fig 6C) For feeding during the light phase CNO (1mg/kg; Sigma) was injected and food was removed 45 minutes before being returned and measured at two hours and four hours (Fig 6F).

Saccharin and Sucrose Preference Tests

Mice were placed in feeding cages (BioDaq) and given ad lib access to standard chow and water for 4 days during the acclimation period. During the saccharin preference test mice had access to water on one side of the cage and saccharin (0.1% w/v, Sigma) on the other. Sides of the bottles were reversed after two days. Total intake of saccharin and water was recorded for 4-5 days. Before beginning the sucrose preference test, there was a 2-day washout period where mice only had access to water and chow. During the sucrose preference test mice had access to saccharin (0.1% w/v, Sigma) on one side of the cage and sucrose (4% w/v, Colonial Sugar Refining Company) on the other. These concentrations were chosen as they had previously been shown to be of similar sweetness and are iso-prefered by mice (Bachmanov et al., 2001). Mice had 3 days access to sucrose and saccharin. On the 3rd night food was removed from the cage and intake of sucrose and saccharin were recorded over the fasting period.

High Fat/High Sugar Diet Preference

Following the sucrose preference test in the feeding cages and a second washout period, mice were given *ad libitum* access to chow in one food hopper, and high fat/high sugar chow (HFD) (23% fat, Specialty Feeds) in the other. Food intake was recorded for 4 days. On the 4th night access to the food hoppers was blocked at 5pm before being opened again at 9am the following day. Food intake in response to the overnight fast was recorded for 24 hours.

Statistical Analysis

Data are expressed mean with standard error of the mean. Comparisons were tested using two-tailed unpaired t-tests or paired t-tests. A two-way ANOVA with Sidak's multiple comparison test was applied when there both fasting and fed conditions, or when a variable was measured over time. $p<0.05$ was considered statistically significant. Graphs were generated in Prism (Version 8.4, 2020).

3.3 Results

Chronic circuit ablation

Initially, we confirmed the presence of dense monosynaptic mPFC-LH projections in mice using the retrograde tracer Cholera toxin B (CTb) injected bilaterally in to the LH (Fig 1A). Significant uptake of the red fluorophore is observed throughout mouse mPFC, which is comprised of the prelimbic cortex (PrL) and infralimbic cortex (IL) (Fig 1B). We then defined our mPFC target region as the intersection of the PrL and IL as this is where we observe LH projection neurons. To chronically ablate the mPFC-LH circuit we used a dual viral approach (Fig 1D). Cav2-cre (del Rio et al., 2019) was used to insert cre-recombinase into mPFC-LH projection neurons and a genetically engineered cre-dependent caspase virus was then targeted to these neurons (AAV-flex-tacasp3-TEVp), which when expressed results in caspase-mediated apoptosis as previously described (Yang et al., 2013). We confirmed cell ablation by staining for the neuronal marker NeuN and determining % area of cells in the mPFC (Fig 1E). Caspase expression reduces % cell area in the mPFC-LH group by approximately 10% (Fig 1E). mPFC-LH circuit ablation increases % body weight gain on a chow diet over the 14-week experimental period (Fig 1F). Despite this, there were no detectable differences in chow intake (Fig 1I) suggesting increased body weight was not due to increased food intake – at least over a short-term period. When mice were given access to a palatable high fat/high sugar diet (HFD) and chow in BioDaq feeding cages all mice consumed only the HFD indicating the mPFC-LH circuit is unlikely to influence food preference when both high value (high fat/high sugar) and low value (chow) options are available. Circuit ablation increases the amount of HFD consumed over a 4-day period (Fig 1I&K) and this is not due to differences in body weight (Fig 1J), suggesting this circuit may specifically influence the consumption of palatable food. To determine whether the mPFC-LH circuit is required for the feeding response to a homeostatic challenge we performed fasting-refeeding experiments during both chow and HFD consumption periods. Circuit ablation had no effect on chow intake following an overnight fast (Fig 1G&H), or HFD following an overnight fast (Fig 1 L-M) suggesting the homeostatic drive to eat operates independently of this circuit, although there was an interaction between circuit ablation and cumulative HFD intake over time during the HFD refeeding period (Fig 1N) suggesting that circuit ablation influences the rate at which the homeostatic deficit is corrected over the light/dark cycle. From this set of feeding experiments we conclude that the mPFC-LH circuit is likely an anorexogenic circuit as ablation results in increased body weight and increased palatable diet intake, however fasting-refeeding experiments demonstrate this circuit does not influence the appropriate feeding response to an acute homeostatic challenge.

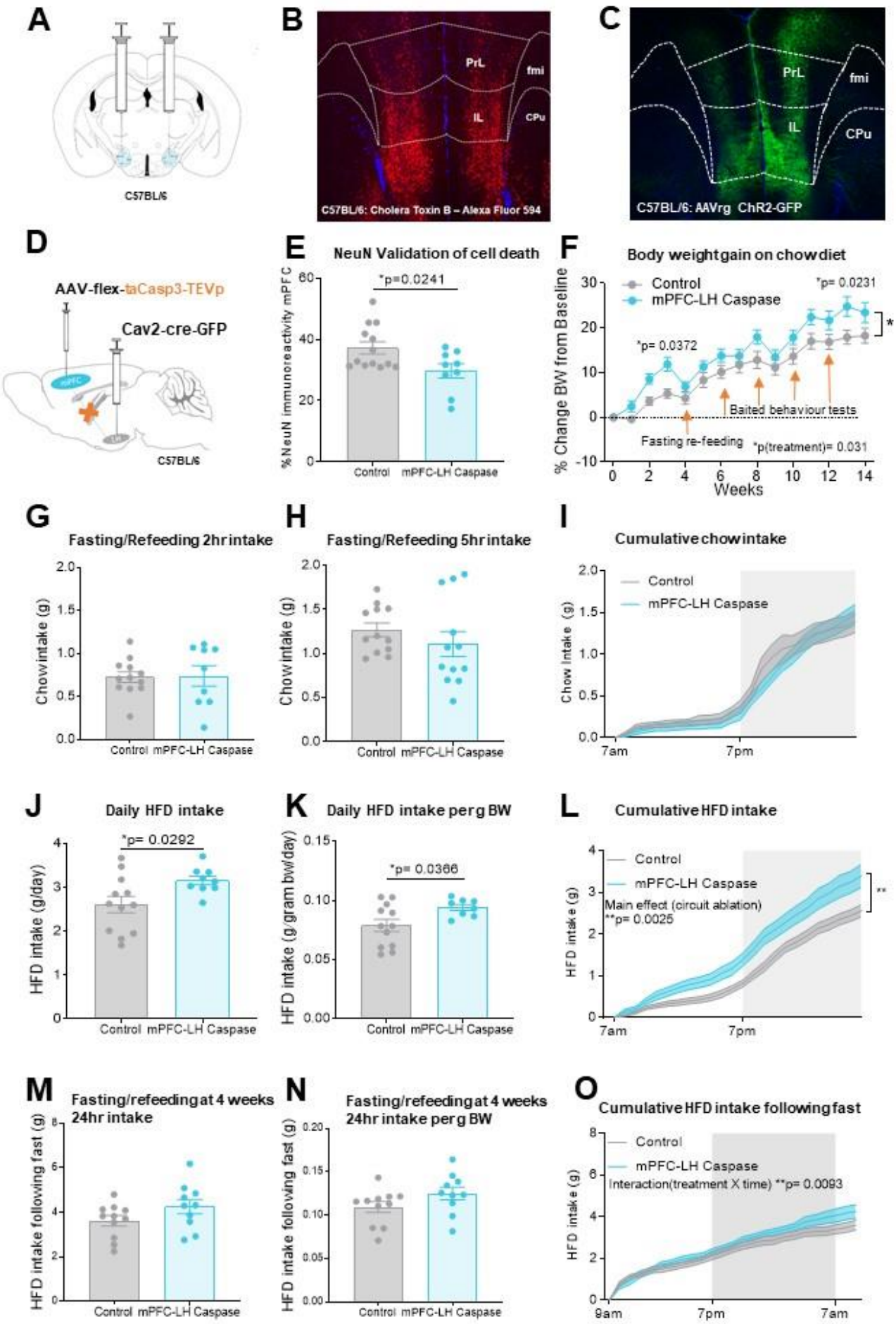

A
C57BL/6
B
PrL
fmi
IL
CPu
C57BL/6: Cholera Toxin B – Alexa Fluor 594
C
PrL
fmi
IL
CPu
C57BL/6: AAVrg ChR2-GFP
D
AAV-flex-taCasp3-TEVp
Cav2-cre-GFP
mPFC
LH
C57BL/6
E
NeuN Validation of cell death
*p=0.0241
% NeuN immunoreactivity mPFC
Control
mPFC-LH Caspase
F
Body weightgain on chowdiet
Control
mPFC-LH Caspase
*p= 0.0231
*
*p= 0.0372
Baited behaviour tests
Fasting re-feeding
*p(treatment)= 0.031
% Change BW from Baseline
Weeks
G
Fasting/Refeeding 2hr intake
Chow intake (g)
Control
mPFC-LH Caspase
H
Fasting/Refeeding 5hr intake
Chow intake (g)
Control
mPFC-LH Caspase
I
Cumulative chow intake
Control
mPFC-LH Caspase
Chow intake (g)
7am
7pm
J
Daily HFD intake
*p= 0.0292
HFD intake (g/day)
Control
mPFC-LH Caspase
K
Daily HFD intake per g BW
*p= 0.0366
HFD intake (g/gram bw/day)
Control
mPFC-LH Caspase
L
Cumulative HFD intake
Control
mPFC-LH Caspase
Main effect (circuit ablation)
**p= 0.0025
**
HFD intake (g)
7am
7pm
M
Fasting/refeeding at 4 weeks
24hr intake
HFD intake following fast (g)
Control
mPFC-LH Caspase
N
Fasting/refeeding at 4 weeks
24hr intake per g BW
HFD intake following fast (g)
Control
mPFC-LH Caspase
O
Cumulative HFD intake following fast
Control
mPFC-LH Caspase
Interaction(treatment X time) **p= 0.0093
HFD intake (g)
9am
7pm
7am

Figure 1. Chronic ablation of mPFC-LH circuit results in body weight gain and increased high fat/high sugar diet consumption. The retrograde tracer Cholera-toxin B (CTb) conjugated with Alexa fluorophore 594 or Retrograde AAV ChR2-GPF was injected bilaterally into the LH of C57BL/6 mice (A). A representative image shows the transport of the red fluorophore throughout the mouse mPFC, which is comprised of the infralimbic (IL) and pre-limbic (PrL) cortex (B). A representative image shows expression of the reporter protein GFP throughout the PrL and IL (C). The retrograde canine adenovirus-2 encoding for Cre-recombinase (CAV2-cre-GFP) was injected bilaterally into the lateral hypothalamus of C57BL/6 mice before a cre-dependent AAV construct encoding for a genetically engineered caspase-3 was injected bilaterally into the mPFC, at the intersection of the IL and PrL, where LH projection neurons were observed (D). Caspase injection reduced % area of immunoreactivity of the neuronal marker NeuN in the mPFC (E; n=9-13, Student's unpaired t-test). Circuit ablation resulted in increased % bodyweight gain on a chow diet (F; n=9-13, two-way ANOVA with Sidak's multiple comparison test, orange arrows indicate where mice underwent fasting for feeding or baited behaviour experiments – light-dark box or open field). Circuit ablation did not affect food intake at 2 hours (G) or 5 hours (H) following an overnight fast at weeks following surgery. There was no difference in chow intake over a four-day period in Biodaq feeding cages between groups (I). Circuit ablation increased consumption of a high fat/high sugar diet over a four-day period (J; n=8-12, Student's unpaired t-test) and consumption of a high fat/high sugar diet per gram body weight (K; n=8-12, Student's unpaired t-test). Cumulative high fat/high sugar diet intake was greater in the circuit ablation group (L; n=8-12, two-way ANOVA). Circuit ablation did not affect high fat/high sugar diet consumption following an overnight fast (M-N), though there was an interaction between circuit ablation and high fat/high sugar cumulative intake over time following the fast (O; n=8-12, two-way ANOVA). BW– body weight; Cpu – Caudate putamen; fmi – forceps minor of the corpus callosum; GFP- green fluorescent protein; HFD – high fat/high sugar diet; IL – infralimbic cortex; mPFC – medial prefrontal cortex; PrL – pre limbic cortex. Grey shading in I,L & O indicates dark phase.

Given that LH circuits are known to influence anxiety behaviours and locomotor activity (Cassidy et al., 2019; Patterson et al., 2015; Qualls-Creekmore et al., 2017; Woodworth, Batchelor, et al., 2017) we tested mice in the elevated plus maze (EPM) (Fig 2A) and open field (Fig 2D) to determine whether ablation of the mPFC-LH might influence these potentially confounding factors. Circuit ablation has no effect on % time spent in the aversive open arms of the EPM, but reduces entries into the open arms (Fig 2B&C) suggesting the mPFC-LH circuit has some influence over exploratory behaviour. There were no differences in velocity or distance travelled in the open field (Fig 2E&F) indicating that mPFC-LH circuit ablation does not affect locomotor activity.

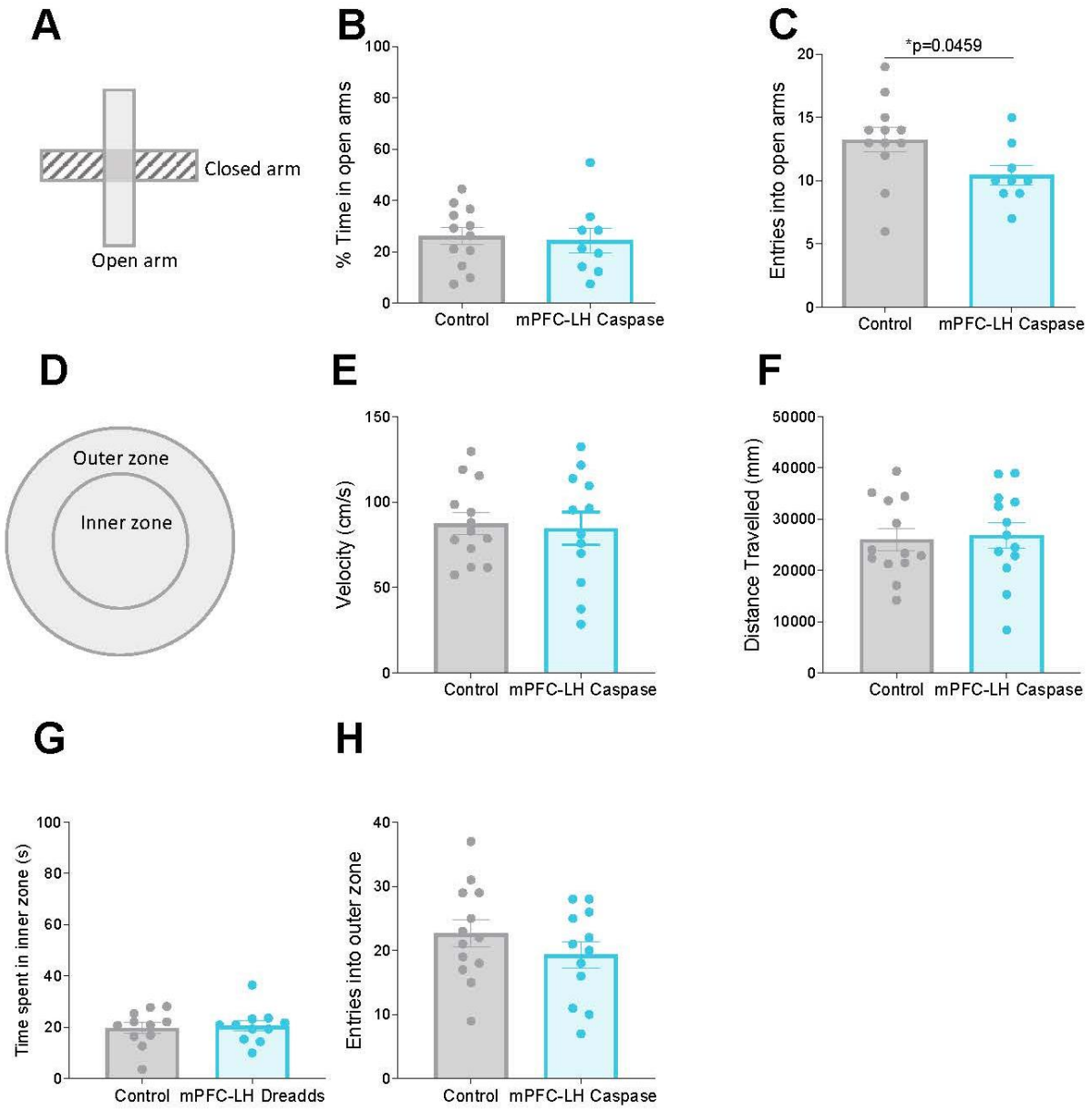


Figure 2. Chronic ablation of mPFC-LH circuit has no effect on anxiety-like behaviour or locomotor activity. Elevated plus maze apparatus (A). Chronic circuit ablation had no effect on %time spent in open arms (B) but reduced entries into open arms (C; n=9-12, Student's unpaired t-test). Open field apparatus (D). Chronic circuit ablation had no effect on velocity (E), distance travelled (F), %time spent in the inner zone (G) or entries into the outer zone in the open field. Data are presented mean ± SEM.

The mPFC is understood to elicit top down restraint of reward seeking via projections to the nucleus accumbens (NAc) (Kim et al., 2017). To test whether ablation of the mPFC-LH circuit would increase risk taking behaviour to obtain a palatable food reward we used baited anxiogenic environments (Lockie et al., 2017)(Fig 3A&G). mPFC circuit ablation has no effect on the amount of peanut butter pellet (bait) consumed (Fig 3B) or any behavioural variable assessed in the baited light-dark box (Fig 3B-F). As expected, fasted mice consume more of the peanut butter pellet (Fig 3B) and spend more time in the risky food zone, make more entries into the food zone and spend more time in the light chamber than mice in the fed state (Fig 3C-E), illustrating the effect hunger has on behaviour. Circuit ablation also does not affect peanut butter pellet intake in the baited open field (Fig 3H) or any behaviour assessed during the test (Fig 3I-L). Again, fasting increases pellet consumption (Fig 3H), time in the risky food zone, entries into the food zone, and time in the

inner zone Fig (3I-K). Together our results suggest the mPFC-LH circuit does not influence palatable food seeking in a risky environment in either the fed or fasted state, therefore supporting results from earlier feeding experiments that suggest the mPFC-LH circuit is not required to maintain either the appropriate behavioural or feeding response to acute homeostatic challenges.

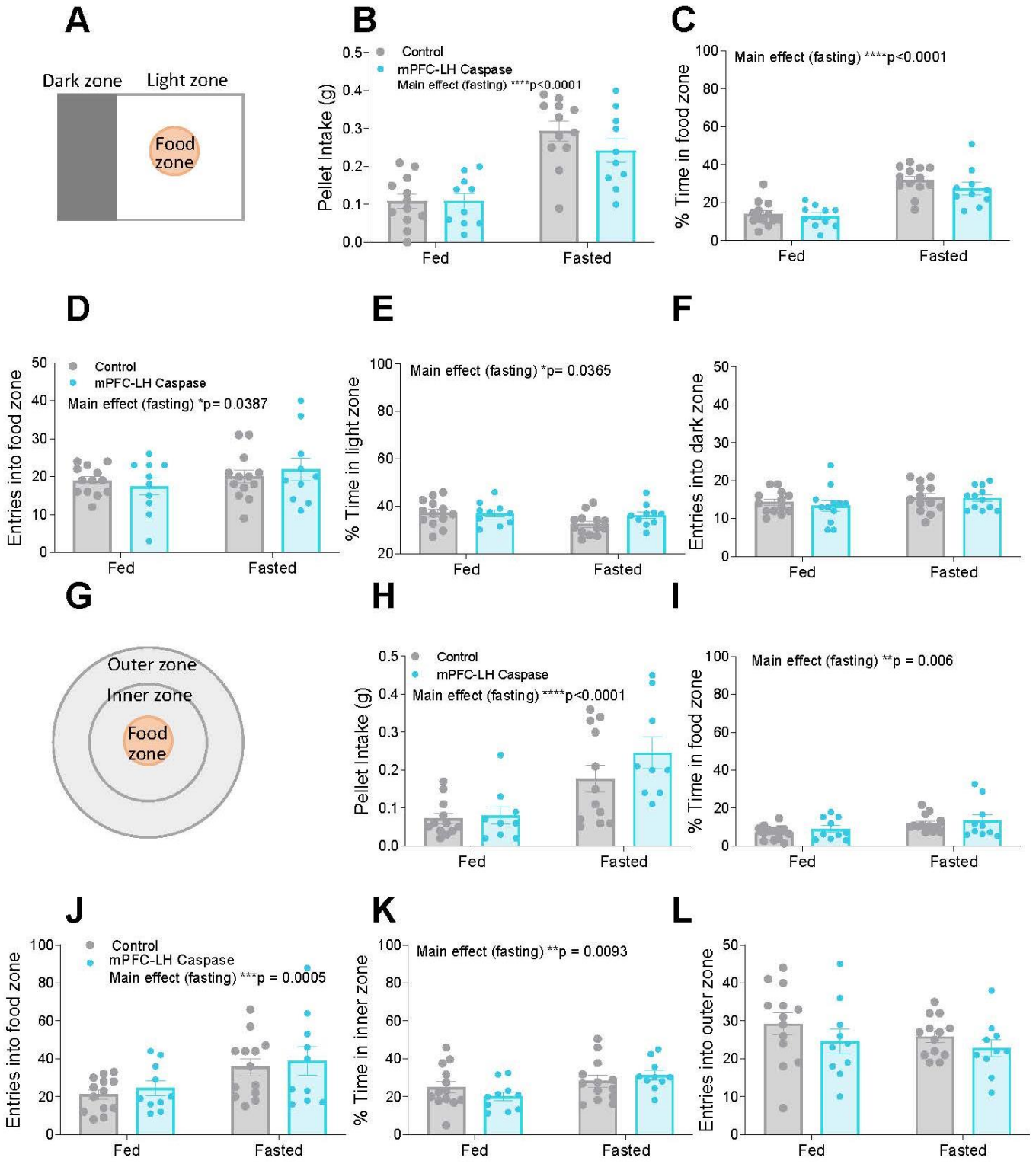


Figure 3. Chronic ablation of mPFC-LH circuit has no effect on risk-reward behaviour as assessed by baited behavioural tasks. Baited light-dark box: One peanut butter pellet was placed in the centre of the

light zone as indicated by the food zone (A). Chronic circuit ablation had no effect on pellet intake, %time spent in food zone, entries into food zone or %time in light zone, however there was a main effect of metabolic state (fed vs fasted; B-E; n=10-13, two-way ANOVA with Sidak's multiple comparison test). Chronic circuit ablation and metabolic state had no effect on entries into dark zone from light zone (F). Baited open field: 3 peanut butter pellets were placed in the centre of the inner zone as indicated by the food zone (G). Chronic circuit ablation had no effect on pellet intake, %time spent in food zone, entries into food zone or %time in inner zone, however there was main effect of metabolic state (fed vs fasted; H-K; n=10-13, two-way ANOVA with Sidak's multiple comparison test). Chronic circuit ablation and metabolic state had no effect on entries into outer zone from inner zone (L). Data are presented mean ± SEM.

To investigate whether mPFC-LH circuit ablation might have differential effects on reward seeking behaviour when mice are challenged with an long-term energy deficit, we chronically food deprived a separate cohort of mice to 85 % of their body weight with the mPFC-LH projecting neurons ablated using the same protocol as described in Fig 1 D. Circuit ablation with calorie restriction has no effect on anxiety like behaviour in the EPM (Fig 4A&B) or locomotor activity in the open field (Fig 4C&D). In the baited light-dark box circuit ablation with calorie restriction does not affect peanut butter pellet intake (Fig 4E) time spent in the risky food zone (Fig 4F), or latency to enter the food zone (Fig 4H). However, calorie restricted mice with mPFC-LH circuit ablation enter the food zone a greater number of times than controls (Fig 4G), which indicates more movement within the risky area of the apparatus, suggesting increased appetitive behaviour, without changes to consummatory behaviour. In the baited open field circuit ablation with calorie restriction has no effect on pellet intake (Fig 4I), time in food zone (Fig 4J), or entries into the food zone (Fig 4K). However, circuit ablation with calorie restriction reduces the latency to enter the food zone (Fig 4L) suggesting mice with mPFC-LH circuit ablation may be less risk aversive, or have less restraint of risk taking behaviour to obtain a food reward, when initially placed in the apparatus. These results demonstrate the need to examine all metabolic states, as it appears when mice are chronically energy deprived the mPFC-LH circuit may influence appetitive or approach behaviours.

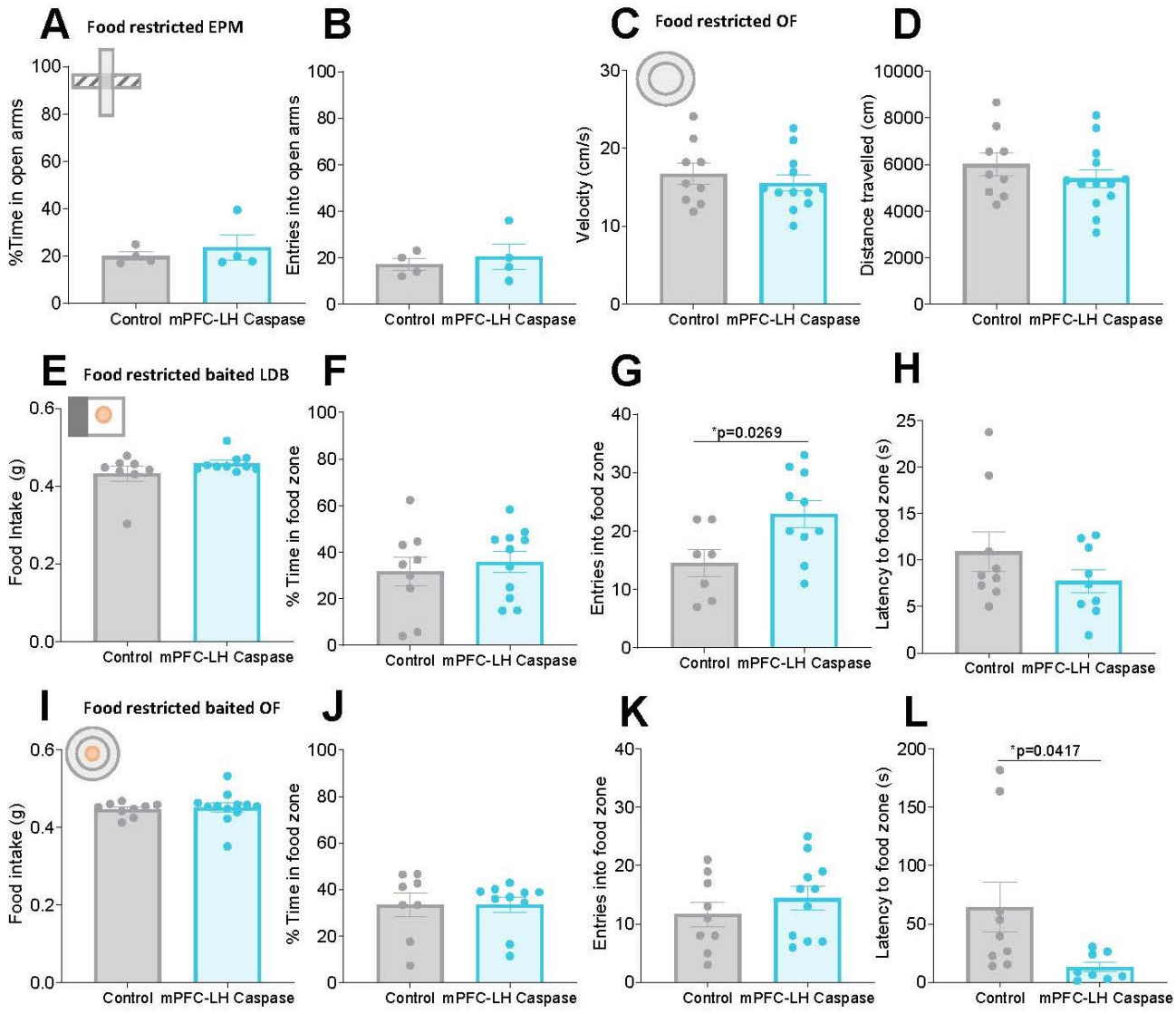


Figure 4. Anxiety-like behaviour and risk-reward behaviour in chronic calorie-restricted mice with chronic ablation of mPFC-LH circuit. Chronic circuit ablation in food restricted mice had no effect on %time spent in the open arms or entries into the open arms in the elevated plus maze (A&B). Chronic circuit ablation in food restricted mice had no effect on velocity or distance travelled in the open field (C&D). There was no effect of chronic circuit ablation in food restricted mice on food intake or %time in food zone in the baited light-dark box (E&F). Chronic circuit ablation in food restricted mice increased entries into food zone in the baited light dark box (G; n=7-10, Student's unpaired t-test) but not latency to reach food zone (H). Chronic circuit ablation in food restricted mice had no effect on food intake, %time in food zone or entries into the food zone in the baited open field (I-K). Chronic circuit ablation in food restricted mice reduced the latency to food zone in the baited open field (L; n=8-9, Student's unpaired t-test). Data are presented mean ± SEM. EPM – elevated plus maze; LDB – light dark box; OF – open field

Given the LH is a key brain region involved in the expression of motivated reward seeking behaviours, including compulsive sucrose seeking (Nieh et al., 2015) we further investigated any role for the mPFC-LH in reward processing through a series of two bottle preference tests comparing preference for sweet non-nutritive (saccharin) and sweet energy containing (sucrose) solutions (Fig 5). Firstly, mice were given access to a saccharin solution and water (Fig 5A). Circuit ablation does not affect saccharin solution consumption (Fig 5C&E) or preference for saccharin solution over water (Fig 5B&D), indicating mice are not anhedonic.

We next asked whether circuit ablation affects the preference for caloric versus non-calorie rewards since reward value assigned to sweet taste and calorie content are mediated through separate neural circuits (Tellez et al., 2016). To investigate any differences in processing of hedonic versus nutritive rewards associated with circuit ablation we gave mice access to a saccharin solution and a sucrose solution in both fed and fasted states (Fig 5F&K). Circuit ablation does not affect sucrose intake (Fig 5H&J) or sucrose preference over saccharin (Fig 5G&I) in a fed state. Given our feeding experiments show that this circuit does not affect chow or high fat/high sugar consumption in response to a fast, we next tested that this circuit would not influence liquid sucrose consumption when mice were deprived of food. As expected, when mice are fasted overnight (food removed but bottles available) sucrose consumption is dramatically increased (Fig 5Q) confirming the appropriate detection of calorie need. Indeed, mPFC-LH circuit ablation does not affect sucrose consumption (Fig 5M&P), energy intake (Fig 5) or sucrose preference over saccharin (Fig 45L&N) during an overnight fast confirming this circuit is not involved in the response to acute homeostatic challenges when given access to liquid sucrose.

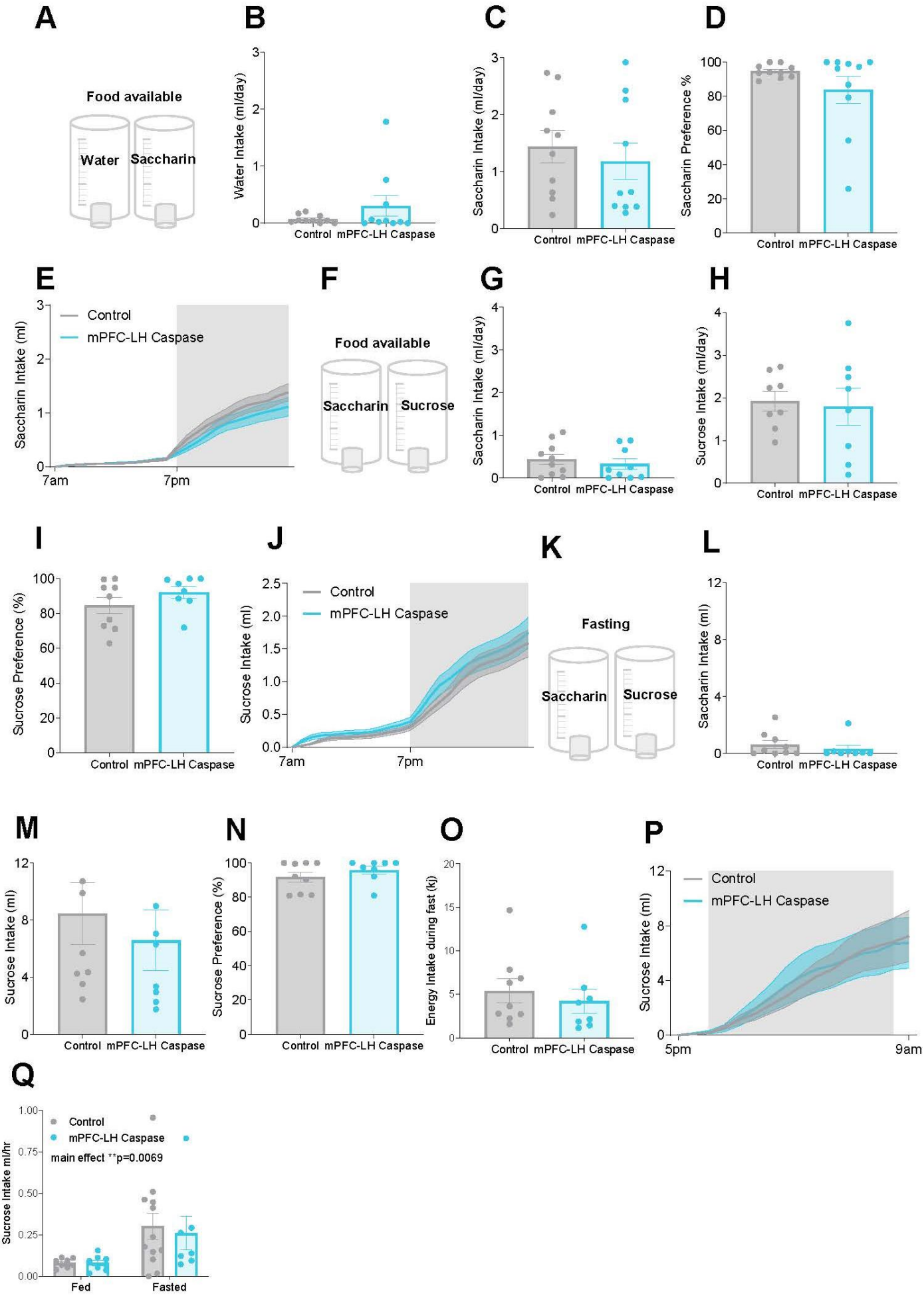

A
Food available
Water
Saccharin
B
Water Intake (ml/day)
Control mPFC-LH Caspase
C
Saccharin Intake (ml/day)
Control mPFC-LH Caspase
D
Saccharin Preference %
Control mPFC-LH Caspase
E
Saccharin Intake (ml)
Control
mPFC-LH Caspase
7am
7pm
F
Food available
Saccharin
Sucrose
G
Saccharin Intake (ml/day)
Control mPFC-LH Caspase
H
Sucrose Intake (ml/day)
Control mPFC-LH Caspase
I
Sucrose Preference (%)
Control mPFC-LH Caspase
J
Sucrose Intake (ml)
Control
mPFC-LH Caspase
7am
7pm
K
Fasting
Saccharin
Sucrose
L
Saccharin Intake (ml)
Control mPFC-LH Caspase
M
Sucrose Intake (ml)
Control mPFC-LH Caspase
N
Sucrose Preference (%)
Control mPFC-LH Caspase
O
Energy Intake during fast (kj)
Control mPFC-LH Caspase
P
Sucrose Intake (ml)
Control
mPFC-LH Caspase
5pm
9am
Q
Sucrose Intake ml/hr
Control
mPFC-LH Caspase
main effect **p=0.0069
Fed
Fasted

Figure 5. Chronic ablation of mPFC-LH circuit has no effect on sucrose or saccharin intake in a two bottle preference task. Mice were given *ad lib* access to bottles containing water and saccharin and *ad lib* access to food for 4 days (A). Chronic circuit ablation had no effect on water intake, saccharin intake, preference for saccharin or cumulative saccharin (B-E). Mice were given *ad lib* access to bottles containing saccharin and sucrose with *ad lib* access to chow for 4 days (F). Chronic circuit ablation had no effect on saccharin intake, sucrose intake, preference for sucrose or cumulative sucrose intake (G-J). Mice were given *ad lib* access to bottles containing saccharin and sucrose during an overnight fast (K). Chronic circuit ablation had no effect on saccharin intake, sucrose intake, energy intake, or cumulative sucrose intake during an overnight fast (L-P). There was no effect of circuit ablation on sucrose intake per hour, although a main effect of metabolic state (fed vs fasted; Q; n= 8-10, two-way ANOVA with Sidak's multiple comparison test). Data are presented mean ± SEM. Grey shading in E,J & P indicates dark phase.

Acute circuit activation with DREADDs

Our results from the chronic circuit ablation studies suggest the mPFC-LH circuit may have an anorexigenic role, since deletion of this circuit resulted in body weight gain and increased consumption of a palatable diet. We therefore predicted that acutely activating the mPFC-LH circuit would inhibit feeding. To activate this circuit, we used a dual viral approach to express the stimulatory DREADD hM3Dq into mPFC-LH projecting neurons (Fig 6A). This resulted in significant expression of the stimulatory DREADDs receptor and the mCherry reporter protein throughout the PrL cortex and IL cortex (Fig 6B). Firstly, to determine whether acute mPFC-LH activation inhibits feeding we examined food intake following a short-term fast during the dark phase, the period when mice consume the majority of their energy intake (Fig 6C). While acute activation of the mPFC-LH circuit does not result in a statistically significant reduction in food intake at 2 hours from food being available (Fig 6D), circuit activation does significantly suppress food intake at 4 hours (Fig 6E). To determine whether mPFC-LH circuit activation also suppresses food intake during the light phase, the period when mice are typically inactive, we tested mice during early hours of the light phase (Fig 6F). Again, while acute activation of the mPFC-LH circuit does not result in a significant reduction in food intake at 2 hours from food being available (Fig 6G), circuit activation does significantly suppress food intake at 4 hours (Fig 6H). Finally, we investigated whether acute mPFC-LH circuit activation could suppress feeding when mice are challenged with a longer term overnight fast (Fig 6I). Acute circuit activation does not significantly suppress food intake at 2 hours (Fig 6J) or 4 hours (Fig 6K) from food being available, despite a strong trend at 2 hours ($p=0.0678$) which is consistent with the idea that this circuit does not influence the feeding response to a homeostatic challenge. Together, this supports the hypothesis that the mPFC-LH circuit is an anorexigenic circuit.

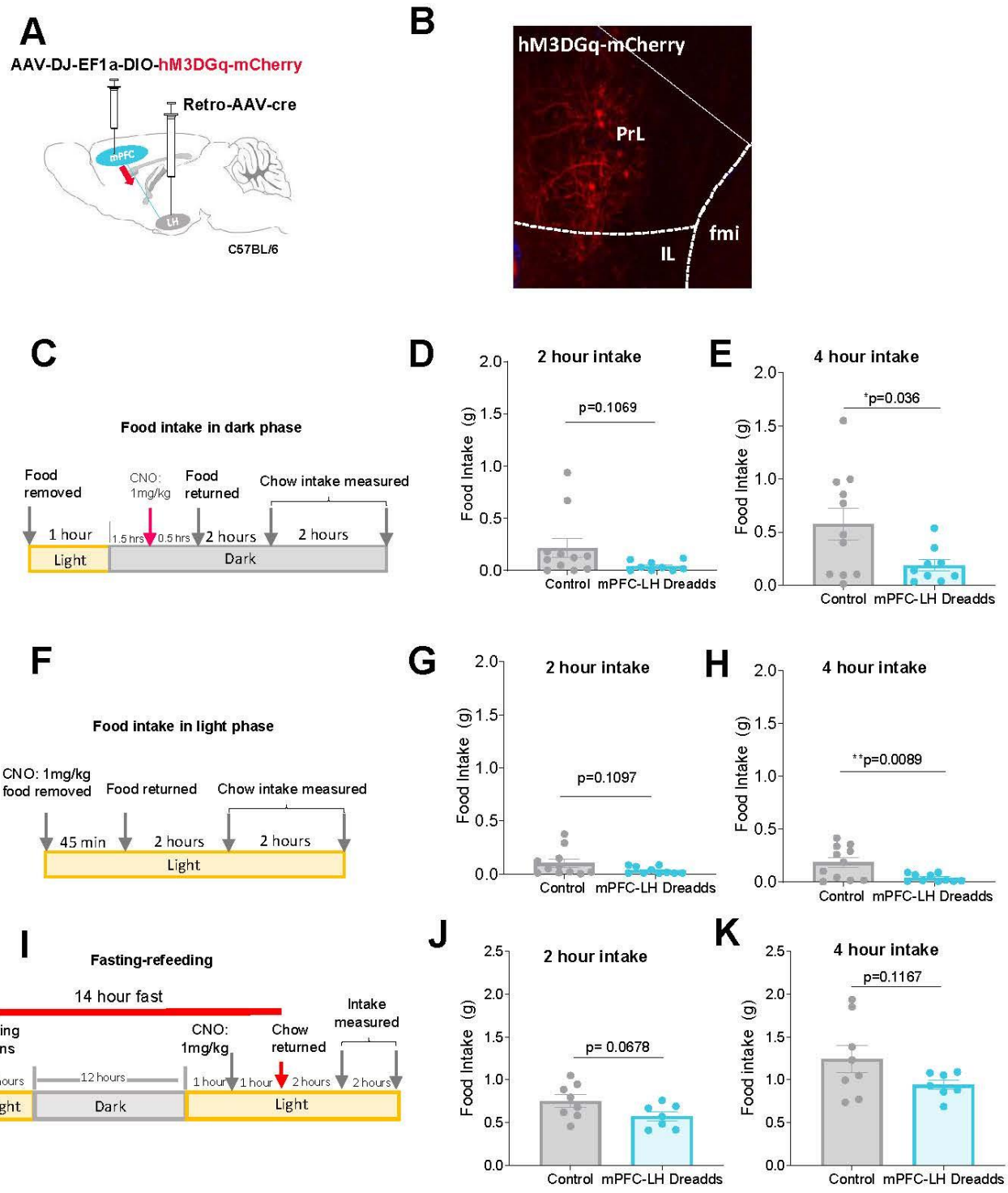


Figure 6. Acute activation of mPFC-LH circuit using Gq DREADDs suppresses food intake. A retrograde AAV virus encoding for cre-recombinase was injected bilaterally into the LH of C57BL/6 mice before a cre-dependent AAV encoding for the human muscarinic receptor hM3Dq and m-Cherry reporter protein was injected bilaterally into the mPFC (A). A representative image shows hM3Dq DREADD-mCherry expression in mPFC-LH projecting neurons (B). Experimental timeline used to assess food intake in the dark phase following mPFC-LH circuit activation with CNO (C). Circuit activation had no significant effect on 2-hour food intake (D; n=9-11, Student's unpaired t-test p=0.11) but suppressed food intake at 4 hours (E; n=9-11, Student's unpaired t-test). Experimental timeline used to assess food intake in the light phase following mPFC-LH circuit activation with CNO (F). Circuit activation had no effect on food intake at 2 hours (G), but suppressed 4-hour food intake (H; n=9-11, Student's unpaired t-test) Experimental timeline used to assess food intake in the light phase following an overnight fast and mPFC-LH circuit activation with CNO (I). Circuit

activation had no effect on food intake at 2 hours (J), or at 4 hours (K) (n=7-8). Data are presented mean ± SEM. CNO - Clozapine-N-oxide.

To determine whether there are any anxiety behaviours or changes to locomotor activity associated with activation of the mPFC-LH circuit we used the EPM and open field (Fig 7A-D). Consistent with our results from the circuit ablation studies, acute circuit activation does not affect anxiety-like behaviour in the EPM (Fig 7A&B) or movement in the open field (Fig 7C&D). In addition, we tested mice in the light-dark box to further assess anxiety-like behaviour (Fig 7E&F). Circuit activation does not affect % time in the light zone (Fig 7E) or significantly affect entries into the light zone (Fig 7F). Thus, the reduction in food intake observed in our feeding experiments is not related to any increase in anxiety-like behaviour.

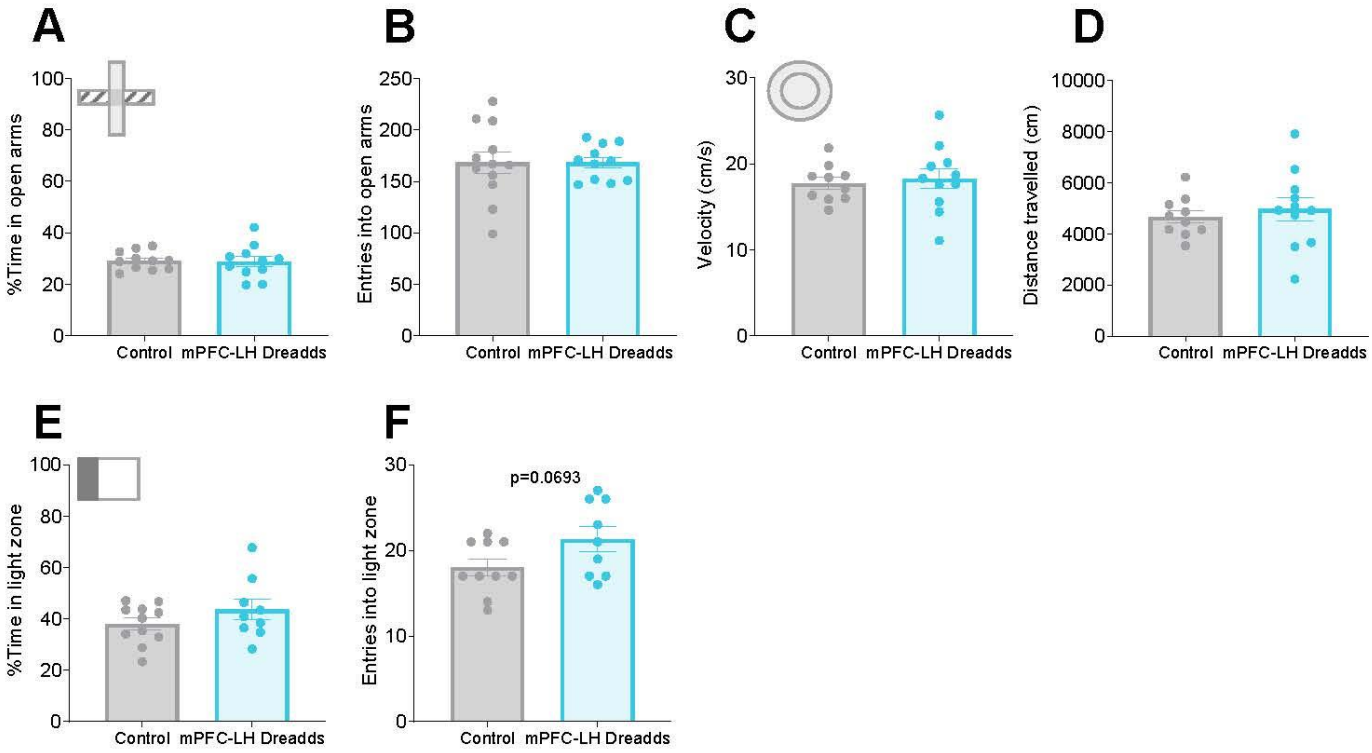


Figure 7. Acute activation of mPFC-LH circuit using Gq DREADDs has no effect on anxiety-like behaviour or locomotor activity. Acute circuit activation had no effect on %time spent in open arms or entries into open arms in the elevated plus maze (A&B). Acute circuit activation had no effect on velocity or distance travelled in the open field (C&D). Acute circuit activation had no effect on %time spent in the light zone or entries into the light zone in the light dark box (E&F). Data are presented mean ± SEM.

To determine whether acute activation of mPFC-LH circuit influences motivated behaviour to obtain a sucrose pellet we tested mice using home cage feeding experimentation devices (FED; Fig 8A). Mice were trained to learn a left nose poke (active side) delivers a sucrose pellet for 7 days from fixed ratio (FR) of one (FR1; one nose poke delivers one pellet) to FR5 until all mice correctly select the active side 75% of total pokes performed (Fig 8B). Initially, we considered whether acute mPFC-LH activation would suppress operant responding, and therefore motivation to obtain a sucrose pellet. Mice were fasted for three hours at the start of the dark phase, following the same protocol as the feeding experiment in the dark phase (Fig 6C), and tested on a progressive ratio task following CNO injection (Fig 8D). Expectedly, fasted control animals nose

poke more frequently (Fig 8E&F), reach higher breakpoints (Fig 8G-I) and receive more pellets (Fig J-L) than animals in the fed state. As we predicted, mPFC-LH circuit activation suppresses operant responding (Fig 8E) and breakpoint reached (Fig 8I), which together can be taken as a proxy for appetitive behaviour. The suppression of operant responding only occurs in the acutely fasted state (Fig 8I&L), when mice ordinarily express increase motivation to obtain a caloric reward. Together with the free feeding experiment during the dark phase (6C) our results suggest that activation of the mPFC-LH circuit suppresses both appetitive and consummatory behaviours following a short-term fast.

We then considered the effect of mPFC-LH circuit activation on extinction of the operant response. Acute circuit activation over a 5-day period reduces the % of pokes to the previously active left nose poke (Fig 8M), but not the total number of pokes or inactive side pokes (Fig 8N&O) suggesting activation of the mPFC-LH circuit interferes with normal goal directed behaviour. After day 5 of extinction, mice displayed a similar number of active and inactive pokes and % active side (Fig M-O).

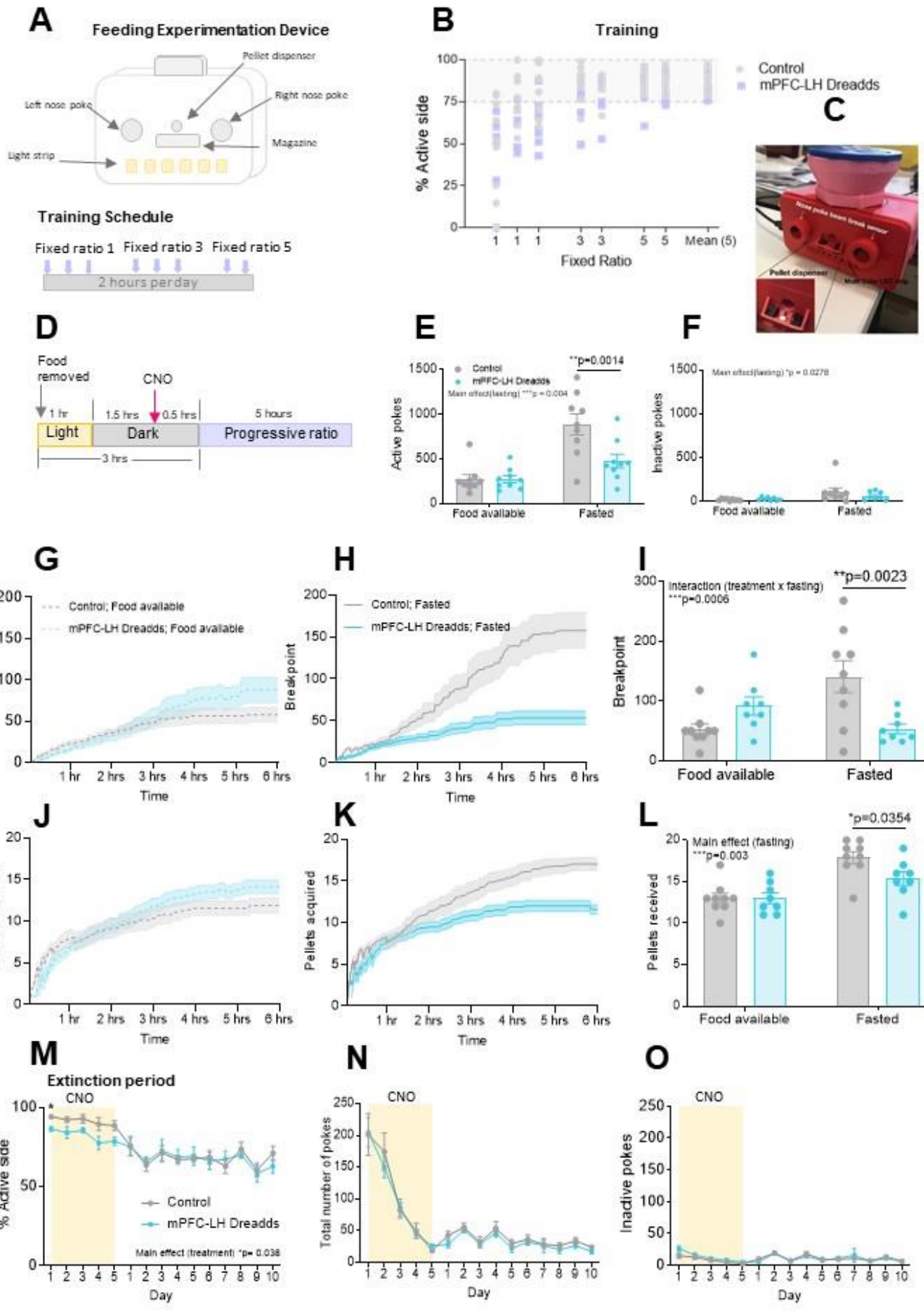

A
Feeding Experimentation Device
Pellet dispenser
Left nose poke
Right nose poke
Magazine
Light strip
Training Schedule
Fixed ratio 1
Fixed ratio 3
Fixed ratio 5
2 hours per day
B
Training
% Active side
Fixed Ratio
Mean (5)
Control
mPFC-LH Dreadds
C
D
Food removed
CNO
1 hr
1.5 hrs
0.5 hrs
5 hours
Light
Dark
Progressive ratio
3 hrs
E
Active pokes
**p=0.0014
Food available
Fasted
F
Inactive pokes
Food available
Fasted
G
Breakpoint
Control; Food available
mPFC-LH Dreadds; Food available
Time
H
Breakpoint
Control; Fasted
mPFC-LH Dreadds; Fasted
Time
I
Breakpoint
Interaction (treatment x fasting)
***p=0.0006
**p=0.0023
Food available
Fasted
J
Pellets acquired
Time
K
Pellets acquired
Time
L
Pellets received
Main effect (fasting)
***p=0.003
*p=0.0354
Food available
Fasted
M
Extinction period
CNO
% Active side
Control
mPFC-LH Dreadds
Main effect (treatment) *p= 0.038
Day
N
CNO
Total number of pokes
Day
O
CNO
Inactive pokes
Day

Figure 8. Acute activation of mPFC-LH circuit using Gq DREADDs influences operant responding to obtain sucrose reward. Feeding experimentation device used to assess operant responding to obtain sucrose pellets (A - top). Mice were trained for 7 days at fixed ratios of 1, 3 and 5 (A- bottom) until mice correctly selected the active nose poke greater than 75% of all pokes (B). Photograph showing feeding experimentation device (C). Experimental timeline for progressive ratio task with CNO with either food removed (fasting) or food available (D). Acute circuit activation reduced the number of pokes on the active side during the progressive ration task (E; n=8-9, two-way ANOVA with Sidak's multiple comparison test). Acute circuit activation had no effect on number of pokes on the inactive side (F). Acute circuit activation had no effect on breakpoint ratio reached in the fed state (G), but reduced the breakpoint ratio reached in the fasted state (H-I; n=8, two-way ANOVA with Sidak's multiple comparison test). Acute circuit activation had no effect on number of pellets received in the fed state (G) but reduced the number of pellets received in the fasted state (H-I; n=8, two-way ANOVA with Sidak's multiple comparison test). During extinction, acute circuit activation reduces the %active nose pokes of total pokes (main effect of control vs mPFC-LH activation) (M; n=8-9; two-way ANOVA with Sidak's multiple comparison test). Acute circuit activation had no effect on number of total pokes or inactive pokes during the extinction period (N-O). Data are presented mean ± SEM. CNO – clozapine-N-Oxide

Acute circuit activation with wireless optogenetics

While the DREADDs approach is useful to determine the effect of acute circuit activation over a short period of time, it does not allow for precise temporal control of circuit activity. Moreover, the DREADD approach in figure 6 highlights the importance of the mPFC-LH pathway but does not offer any molecular insight into the nature of the circuit. Therefore, we employed a novel wireless optogenetics approach to investigate short term manipulations of the vesicular glutamate transporter-1 (Vglut1 cre) expressing neurons in the mPFC-LH circuit activation (Fig 9). Vglut1 is a marker of glutamatergic neurons that is primarily restricted to the cortex (Zeisel et al., 2018). A retrograde cre-dependent virus encoding for hChR2 with H134R mutation was injected bilaterally into the lateral hypothalamus before a wireless optogenetic probe was placed unilaterally above the mPFC (Fig 9A). This allowed us to optogenetically activate the Vglut1 mPFC neurons projecting to the LH (mPFC^{Vglut1}-LH). We confirmed expression of hChR2(H134R) and the mCherry reporter protein throughout the PrL and IL cortex (Fig 9B). Initially, we sought to confirm whether mPFC^{Vglut1}-LH circuit photoactivation would also suppress feeding (D). Consistent with our DREADDs studies, activation of the mPFC^{Vglut1}-LH suppresses feeding (Fig E&F). However, unlike previous feeding experiments, photostimulation of the mPFC^{Vglut1}-LH was sufficient to suppress feeding following an overnight fast. This could be due to the specific downstream targets of the mPFC ^{Vglut1} neurons, and may differ from the actions of the heterogeneous mPFC-LH projection neurons targeted in the DREADDs experiments. This could also be a result of differences in the intensity or temporal nature of photostimulation compared to DREADDs mediated activation – or both.

Next, given extra-hypothalamic inputs into the LH have been demonstrated to be both reinforcing or aversive depending on the location and neuronal population targeted (Jennings et al., 2013, 2015), we considered whether photoactivation of the mPFC^{Vglut1} -LH circuit would influence behaviour in a real-time place preference task as an index of the inherent rewarding or aversive nature of this circuit (Fig G). The time mice spend in the side of the arena paired with photostimulation is reduced in mPFC^{Vglut1}-LH photoactivation group,

compared to a light-off control period (Fig 9H&I), and this is statistically reduced from chance (Fig 9H) and not due to differences in locomotor activity (Fig 9J). This suggests that photoactivation of the mPFC^{Vglut1}-LH circuit is aversive.

We then considered whether photoactivation of the mPFC^{Vglut1}-LH circuit would suppress motivation to obtain a sucrose pellet as we observed with the DREADDs studies. We trained mice in a procedure similar to that described in figure 8B before testing them with a progressive ration task under conditions of photostimulation and no stimulation (Fig 9 L). Photoactivation of the mPFC-LH^{Vglut1} circuit suppresses active nose pokes (Fig 9M), breakpoint ratio reached (Fig 9N), and pellets acquired (Fig 9O) consistent with results from the DREADDs studies. Together our results support a role for the mPFC-LH circuit as an inhibitory controller of feeding and motivated reward-seeking behaviour.

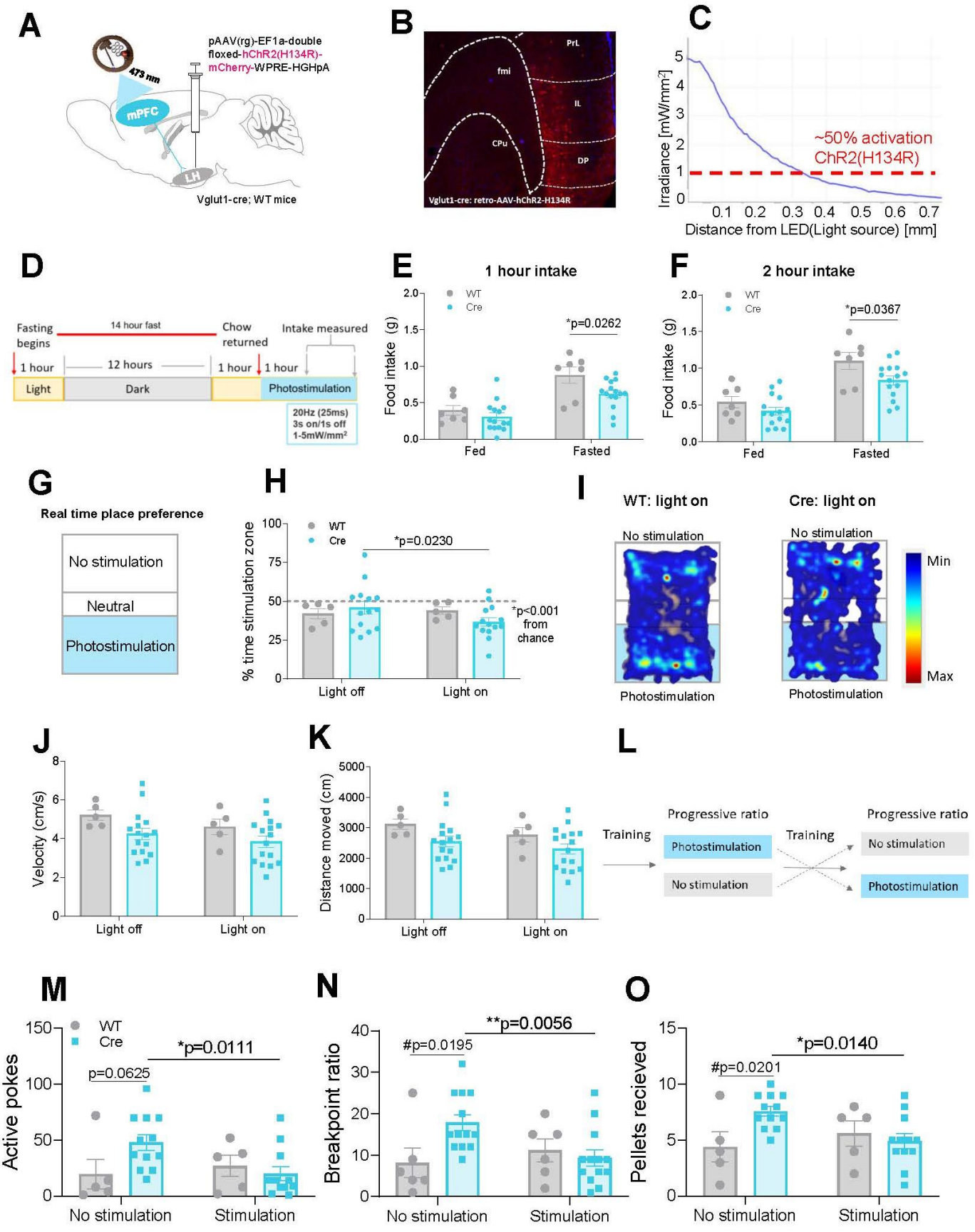


Figure 9. Photostimulation of mPFC^{Vglut1} to LH projection neurons suppresses' food intake and operant responding for sucrose reward and is aversive. A retrograde AAV encoding for a cre-dependent ChR2(H134R) was injected bilaterally into the LH of Vglut-cre expressing mice or wild type mice before a wireless light emitting probe was inserted above the mPFC (A). Expression of ChR2(H134R)-mCherry throughout the mouse mPFC (B). Calculated irradiance values for LED attached to wireless probe (C). Experimental timeline showing fasting re-feeding protocol (D). Photostimulation of mPFC^{Vglut1} to LH projecting

neurons suppressed fasting induced food intake at 1 hour and 2 hours after onset of photostimulation and availability of food compared to wild type controls (E&F; n=7-15, two-way ANOVA with Sidak's multiple comparison test). Real time place preference apparatus – mice were given 10 minutes to explore the apparatus before one side was paired with photostimulation for 20 minutes (G). During the photostimulation (light on) period Vglut-1 cre mice spent less time in the side of the apparatus paired with photostimulation compared to the light off control period (H; n=5-15, two-way ANOVA with Sidak's multiple comparison test) and this was significantly different from chance (n = 15, Student's paried t-test). Example heat maps from wild type and control groups showing arena location in photostimulation non-photostimulation zones (I). Photostimulation did not affect velocity or distance travelled in the real time place preference test (J&K). Experimental protocol for photostimulation during progressive ratio task (L). Photostimulation of $mPFC^{Vglut1}$ to LH projecting neurons reduced the number of nose pokes in the active side (M; n=5-12, two-way ANOVA with Sidak's multiple comparison test); the breakpoint ratio reached (N; n=5-12, two-way ANOVA with Sidak's multiple comparison test); and number of pellets received in the progressive ratio task (O; n=5-12, two-way ANOVA with Sidak's multiple comparison test). Data are presented mean ± SEM. No stimulation vs stimulation: *p<0.05, **p<0.001; WT vs Cre: #p<0.05.

3.4 Discussion

The mPFC is a brain region implicated in a broad range of responses that includes the encoding of cue- and value-association, and decision making related to reward-seeking (Schur et al., 2009). Intriguingly, activation of mPFC neurons can either promote or suppress reward responding depending on the projection specific population targeted, as well as the behavioural paradigm examined (Kim et al., 2017; Otis et al., 2017a). With this in mind it is not surprising that human neuroimaging studies report that the mPFC is hyperactive in conditions of both extreme overweight and extreme underweight suggesting it is likely to be involved in neural processes that override interoceptive, homeostatic signals. While tracing studies show a direct projection from the mPFC to LH – a key site thought to integrate motivational and metabolic information – the role of the mPFC to LH circuit has not been directly tested in the context of feeding and reward. Here, our results suggest that the mPFC to LH circuit is anorexogenic, by suppressing food intake and motivation to consume palatable food. Chronic genetic ablation of the mPFC-LH circuit increases bodyweight on a chow diet and consumption of palatable food. Acute activation of the mPFC-LH circuit and mPFC ^{Vglut1} neurons projecting to the LH using chemogenetics and wireless optogenetics respectively, suppresses food intake and suppresses motivation to obtain palatable food. The mPFC-LH circuit is therefore able to influence both appetitive and consummatory behaviours. It is interesting to note that mPFC-LH circuit ablation did not differentially effect behaviours or consumption in fed or fasted states – suggesting any influence of the mPFC-LH circuit is independent of homeostatic state and demonstrates the mPFC-LH circuit elicits top down control over homeostatic signals.

The mPFC has a number of well-studied projections to the NAc (Kim et al., 2017; Otis et al., 2017a), ventral tegmental area (VTA) (Kim et al., 2017), paraventricular thalamus (PVT) (Otis et al., 2017a) and basolateral amygdala (BLA) (Land et al., 2014). Although mPFC projections to the LH have been described (Barone et al., 1981; Hahn & Swanson, 2010), these have so far received little attention. Consistent with these previous reports we show mPFC neurons project from both the PrL and IL region to the LH. Recently, a study demonstrated acute stimulation of mPFC neurons projecting the LH, the majority of which are excitatory Vglut1-expressing neurons, increased violent attacks against an intruder animal and decreased social signalling prior to an attack, suggesting this circuit might be recruited to quickly modify specific aspects of aggressive behaviour in response to changing social threats (Biro et al., 2018). However, the potential role for the mPFC-LH circuit in feeding and reward seeking was not considered.

We were interested in the PFC-LH projection since recent work with collaborators at Monash University, indicated a direct input pathway from the mPFC to the hypothalamus using spectral dynamic causal modelling in humans (Voight et al 2020, manuscript submitted: *Hunger state and adiposity influence causal interactions between neural systems for feeding, reward and choice*). This approach indicates greater vmPFC to hypothalamic excitation during hunger states compared to satiety states. Intriguingly, our results consistently demonstrate that that acute activation of the mPFC-LH circuit suppresses food intake and motivation for palatable food intake. Accordingly, reduced PFC-LH anorectic tone after chronic circuit ablation increases

body weight gain over time. Thus, our results are not consistent with the findings in humans that vmPFC provides increased excitatory input to the hypothalamus during hunger. One limitation of human imaging studies is the inability to determine which subsets of mPFC neurons might be affected in each disorder, or precisely which mPFC circuits are involved. This makes it hard to predict how a particular circuit may function at a molecular level. For example, in the study considering the role of the mPFC-LH circuit in aggression mentioned above, the authors predicted activation of the mPFC to LH circuit would result in a decrease in the expression of aggressive behaviours based on human data showing an association between structural deficits in the mPFC and increased aggression (Biro et al., 2018). However, similar to us the results of this study contradict the data available from humans. In our case, another potential reason for opposing results is the inability to reliably separate the lateral hypothalamus from other hypothalamic nuclei in human imaging studies. This is particularly important given that rodent tracing studies demonstrate a projection from the PFC to the dorsomedial hypothalamus (Barone et al., 1981). A function role of the PFC to DMH pathway has not been examined and requires investigation.

Important studies utilising optogenetics techniques have identified opposing roles for the genetically defined GABAergic and glutamatergic neurons within the LH (Jennings et al., 2013, 2015; Stuber & Wise, 2016). Photostimulation of LH^{Vgat} neurons, which are molecularly distinct from orexin and melanin concentrating hormone (MCH) expressing neurons, increases consumption of food in fed animals, and increases appetitive behaviours, while photoinhibition has the opposite effect (Jennings et al., 2015). In contrast, photostimulation of LH^{Vglut2} neurons suppresses food intake in fasted animals and is aversive (Jennings et al., 2013). Further supporting these studies, genetic ablation of LH^{Vglut2} neurons increases body weight gain and calories consumed on a palatable diet (Stamatakis et al., 2016). Our chemo-, optogenetic and circuit ablation approach directly phenocopy studies manipulating LH^{Vglut2} neurons. Therefore, we predict that LH^{Vglut2} neurons are a primary downstream target of excitatory mPFC^{Vglut1} LH projecting neurons. Interestingly, the suppression of food intake seen after photostimulation of LH^{Vglut2} neurons (Jennings et al., 2013) or mPFC^{Vglut1}-LH neurons (present study) contrasts with older pharmacological studies, in which glutamate or glutamate receptor agonists injected into the LH elicits feeding (Stanley, Willett III, et al., 1993). These differences are mostly likely due to the increased specificity in control over neuronal firing and neural circuits offered by optogenetics.

However, in line with our results, studies investigating pharmacological inhibition of the mPFC report increased feeding and motivation to obtain food rewards (Mena et al., 2011, 2013). Intra-mPFC injection of DAMGO, a μ-opioid receptor agonist, induces feeding, increases progressive ratio responding for sucrose rewards and increases locomotor activity (Mena et al., 2013). Since μ-opioid receptor agonism produces hyperpolarisation and neuronal inhibition (Al-Hasani & Bruchas, 2011, p.), these results suggest inhibition of a subset of mPFC neurons (those that express the μ-opioid receptor) is sufficient to drive food intake, consistent with our observations herein. Another finding from this study was that intra-mPFC injection of DAMGO increases fos expression in orexin neurons (Mena et al., 2013). This indicates the presence of an indirect circuit from the mPFC to LH, as inhibition of a direct mPFC to LH circuit would not produce an increase

in fos expression in LH orexin neurons. The additional nodes connecting the μ-opioid receptor expressing mPFC neurons to the LH orexin neurons are not investigated in this study (Mena et al., 2013). There is evidence that the lateral septum is one relay point connecting the mPFC to the LH (Carus-Cadavieco et al., 2017). Gamma-oscillations driven by the mPFC are delivered to LH GABA neurons via inhibitory lateral septum somatostatin neurons, and were found to promote appetitive behaviour and improve performance on a learned food reward task (Carus-Cadavieco et al., 2017). Thus, there is both a direct PFC-LH pathway, which we show here to be anorectic and reward suppressing, as well as indirect pathways from the mPFC to LH, both of which described promote food intake, reward seeking and cue-potentiated feeding. Whether this is the case for all indirect pathways from the mPFC to LH is not yet clear, but an interesting idea as an overall commonality shared between indirect mPFC to LH circuits.

An interaction between the PFC and LH has been well-described in a number of studies by Petrovich and colleagues (Anderson & Petrovich, 2018; Cole et al., 2020; Cole, Hobin, et al., 2015; Cole, Mayer, et al., 2015; Petrovich, 2013), in which mPFC neurons are required for cue-potentiated feeding to increase food motivation and food cue memory. Similar to results from Mena *et* al 2013 described above, this involves orexin neurons in the LH and intriguingly a recent study by Cole *et al* 2020) show that orexin projections into the mPFC are required for cue-driven food consumption (Cole et al., 2020). Although the mPFC and LH maybe be key components of a cue-potentiated feeding network, there is currently no evidence that this is through a direct PFC-LH pathway. In fact, our data show that activation of a direct mPFC-LH pathway suppresses food intake and reward seeking.

There is often conflicting data on the role of the mPFC in reward seeking, with numerous studies describing a role in cue-dependent learning, value association and motivation to seek reward, whereas several other studies show mPFC activity is important to suppress reward seeking. For example, a number of studies either using inhibition or activation of the PrL and IL, or genetic or projection-specific populations of neurons that exist across both regions, show that mPFC activity is associated with inhibition of motivated, natural and drug-related reward seeking (Chen et al., 2013; Ferenczi et al., 2016; Jonkman et al., 2009; Kim et al., 2017; Pfarr et al., 2015). However, there are several studies that report opposing results - instead mPFC activity drives responding for natural and drug-related rewards and also reinstatement of cue-induced or context-induced reward seeking following extinction (Bossert et al., 2012; Land et al., 2014; McFarland et al., 2004; Otis et al., 2017a). Otis *et al* 2017 show that these differences can be broadly explained by the projection targets of the mPFC neurons in question, in which corticostriatal pathways increase reward (sucrose in this case) seeking and corticothalamic pathways inhibit reward seeking (Otis et al., 2017a). While the mPFC-LH projections were not investigated in this study our results fit within this simplified model of mPFC circuits. Supporting this idea and relevant to our feeding experiments herein, photoactivation of mPFC D1R neurons, that project to both striatal and limbic regions, promotes food intake and responding for food reward (Land et al., 2014). While the corticostriatal circuit was not explicitly examined in this study, the activation of the corticolimbic neurons was sufficient to increase food intake (Land 2014). Other studies have demonstrated that activation of corticolimbic circuits promotes reward seeking (Malvaez et al., 2019). Possibly, mPFC limbic

and striatal projections have similar roles in promoting reward seeking, while thalamic and hypothalamic projections generally act to suppress motivated behaviours. Although it is important to note that within each projection specific population of mPFC neurons (striatal or thalamic) there is diversity in activity profiles in response to cues that predict reward (Otis et al., 2017a). While it may be possible to generalise about the role of mPFC projections, there are likely smaller subsets of neurons with in each projection specific population that have opposing functions. For example, a subset of mPFC-NAc projection neurons are responsive to foot shock, encode aversion and inhibit reward seeking (Kim et al., 2017). The availability of tools that allow for targeting of specific neuronal populations based on activity response profile provides a powerful technique that will be valuable in further elucidating the role of mPFC circuits.

Beyond the influence of PFC neurons, other cortical inputs into the LH also affect feeding and behaviour. Wu *et al* (2020) investigated right anterior insula cortex inputs into the LH and found that acute activation of this circuit inhibits free-access feeding and is aversive, while chronic inhibition of the circuit increases body weight (Wu et al., 2020). This study also demonstrates that the right anterior insula cortex excitatory neurons are active in response to aversive visceral stimuli, and propose a role for this circuit in the inhibition of homeostatic signals and the drive to eat under emergency situations (Wu et al., 2020). These results are strikingly similar to ours and indicate direct cortical inputs to the LH provide anorectic and aversive information that is independent of homeostatic drive. In our study, activation of the mPFC^{Vgut1} LH projection neurons induces aversive behaviour in a real-time place preference assay. Taken together, this suggests direct cortical to LH information might act as an immediate brake over LH motivational properties and provide top down control over homeostatic drive in response to aversive cues. It was interesting to note that despite observing expression of aversion behaviours in the real-time place preference task, we did not detect difference in anxiety-like behaviour or locomotor behaviour using either circuit ablation or DREADD induced activation of PFC-LH neurons. While the mechanisms underlying these discrepancies are unknown, it presumably reflects differences in acute transient photostimulation of mPFC^{vglut1}-LH neurons versus chronic ablation or longer-term CNO induced activation with DREADDs.

One limitation of our study is that we have not yet investigated whether the mPFC-LH projecting neurons only project to the LH or whether the same neurons have collateral projections to other regions, such as the NaC or the BLA that are involved in both feeding and reward seeking. Tracing studies have shown that some mPFC neurons that project subcortical regions such as the striatum also send axon collaterals to cortical and other subcortical regions (Ferino et al., 1987). However, Biro et al 2018 report that the projections from the mPFC to the LH are largely distinct from the mPFC projections to the medial basal hypothalamus (Biro et al., 2018), suggesting a sole one-to-one projection from the PFC-LH, without collateralisation. A similar observation has been made with other neurons regulating food intake. For example, Agrp neurons, which increases food intake, contain subpopulations of neurons that project to specific downstream targets without collaterisation (Betley et al., 2013). Thus, the lack of collaterisation of neural circuits involved in feeding is not unprecedented, however future experiments to directly confirm this in the mPFC-LH circuit are planned. This

will be achieved by transducing ChR2 into mPFC^{Vglut1} neurons and photostimulating nerve terminals in the LH, as well as examining ChR2 projecting fields.

Human neuroimaging studies from our collaborators and others implicate hyperactivation of the mPFC in obesity (Schur et al., 2009; Stoeckel et al., 2008b) which in contrast to other studies showing that hyperactivation of the mPFC is a feature of anorexia (Ellison et al., 1998b; Uher et al., 2004). Here, our results support a role for hyperactivation of the mPFC to LH circuit in anorexic type behaviours. A limitation of human imaging studies mean that it is not possible to determine which subsets of mPFC neurons might be affected in each disorder, or precisely which mPFC circuits are involved (direct or indirect). We propose that direct cortical to LH circuits are likely to be anorexic in nature, with indirect circuits likely arising from striatal or limbic inputs to the LH acting to promote food intake and reward seeking.

4. Chapter Four: ACC inputs into the LH suppress feeding and increase locomotor activity

4.1 Introduction

Obesity is a complex disease that ultimately arises when energy intake exceeds energy requirements due to both genetic and environmental determinants. Obese patients often report that despite their best intentions, they are unable to adhere to dietary restrictions that are necessary to return to and maintain a healthy weight (Puhl et al., 2008). While there has been extensive research into the neural control of homeostatic food intake, we still know very little about the neural circuits that allow for the overconsumption of energy dense foods in the absence of homeostatic need.

Neuroimaging studies in obese and binge eating disorder patients consistently demonstrate differential activity in the anterior cingulate cortex (ACC) in response to food or food cues (Brooks et al., 2013; De Ridder et al., 2016; Dimitropoulos et al., 2012; Harding et al., 2018; Martin et al., 2010; Murdaugh et al., 2012; Stoeckel et al., 2008). These studies overwhelmingly find that obese individuals, compared to healthy weight controls, have increased activity in the ACC when looking at images of food or tasting, smelling or consuming foods (Brooks et al., 2013). In addition, altered ACC activity is a common feature in obese people with or without food addiction and is thought to integrate both hedonic information related to the value of a food and often opposing, homeostatic or visceral information about metabolic state (De Ridder et al., 2016).

Despite a clear role for the ACC in human obesity, there have been very few animal studies assessing the role of the ACC in feeding behaviour and food-related reward seeking. One recent study reports that disruption of excitatory neuronal signalling in the ACC via either chemogenetic activation or inhibition suppresses operant responding for sucrose pellets (Hart et al., 2019). Calcium imaging in this study identifies separate subsets of ACC neurons that are active either prior to operant responding or during sucrose pellet retrieval, which may explain why both activation and inhibition of the ACC excitatory neurons yields similar results (Hart et al., 2019). In addition, this suggests that specific subsets of ACC neurons may be sensitive to either appetitive or consummatory behaviours (Hart et al., 2019). In support of this idea, it appears that parvalbumin (PV) expressing interneurons of the ACC are active immediately following reward consumption in a foraging task; while a subset of somatostatin (SS) expressing interneurons increase activity during appetitive reward approach (Kvitsiani et al., 2013) – however the behavioural response to manipulations of these interneurons was not investigated here. Another study reports that the ACC is required for normal food foraging and decision making behaviour in a social setting, as pharmacological lesions in this region increases the consumption of highly palatable, rewarding food foraged and a simultaneous decrease in normal social foraging behaviour (Zhong et al., 2017). However, preference for palatable food over chow, and total amount of food consumed during free-feeding was not affected by ACC lesions (Zhong et al., 2017).

Together, the limited number of animal studies in this area suggest a role for the ACC in the normal valuation of a food reward – though this is dependent on the context through which this is investigated.
One factor contributing to obesity may be reduced sensitivity to interoceptive satiety signals that should be integrated when weighing up the reward value of a food (Simmons & DeVille, 2017; Willem et al., 2019). The ACC has dense inputs from interoceptive-related brain regions such as the thalamus, somatosensory cortex and insula (Bliss et al., 2016), and therefore may play a key role in facilitating the appropriate behavioural response to satiety and accurately re-valuing high-energy foods. In support of this idea, ablation of glucose sensitive neurons within the ACC results in increased body weight and an impaired blood glucose response to a glucose tolerance test in mice (Hormay et al., 2019).

A potential pathway that might be involved in the ACC mediated response to internal signals of energy balance are projections to the lateral hypothalamus (LH) – a key site involved in food intake and food reward related behaviours (Clarke et al., 2018; Jennings et al., 2013, 2015). Although ACC neurons project directly to the LH this pathway received very little attention. ACC-LH neurons mediate hypotensive responses to acetylcholine infusion into the cingulate gyrus (Pajolla et al., 2001) or disrupt the release of luteinising hormone (Caceres & Taleisnik, 1981), however, a role for ACC-LH projections in food intake and reward has not been investigated.

The ACC is considered crucial to motivation, reward-based decision making and cognitive control – the ability to flexibly modify behaviour towards goals and away from behaviours that could be considered involuntary or automatic, but not in line with current goals (Shenhav et al., 2016). Therefore the ACC is likely involved in the mindful control of feeding behaviour (Dallman, 2010), which may be low in conditions of obesity and high in conditions of underweight such as anorexia. In support of this, human imaging studies also implicate differential activity of the ACC in patients with anorexia (Ellison et al., 1998a; Uher et al., 2004). Given that cortical inputs to the LH from the insula, a cortical region integrating interoceptive information and implicated in human anorexia and obesity, suppress food intake in response to aversive visceral stimuli (Wu et al., 2020), we examined whether ACC inputs to the LH might similarly influence feeding and food reward-related behaviours. We hypothesised that the ACC to LH circuit would elicit top down control over feeding behaviour, reward seeking and body weight.

4.2 Methods

Animals

All experiments were conducted in accordance with the Monash Animal Ethics Committee guidelines. C57BL/6 male mice were obtained from the Monash Animal Services facility. Mice were aged between 8-10 weeks at beginning of experiments. Mice were maintained on a 12-hour light-dark cycle with *ad libitum* access to standard chow (Rat and mouse pellets, Specialty Feeds, Western Australia) and water under standard laboratory conditions (21°C). All mice were group housed following surgery and throughout the duration of behaviour experiments. Mice were individually housed for food intake experiments, feeding cage experiments. Caspase experiments were conducted within the light phase (7am-7pm). DREADDs experiments were conducted through the dark phase with mice on a reverse light cycle (11pm-11am). Mice were handled for 5 min each day for five consecutive days leading up to behaviour experiments.

Viruses and Surgical procedures

Mice were anesthetised using isoflurane (5% for induction, 2% for maintenance) and positioned on a stereotaxic frame (Stoelting). A Neuros syringe (Hamilton, Reno, NV, USA) was used to deliver virus to the target regions; LH (-1.4mm Bregma; +/- 1.0mm lateral; -4.8mm ventral from surface of brain) and ACC (+1.1mm Bregma; +/- 0.3mm lateral; -1.3mm ventral from surface of brain and +1.3 Bregma; +/- 0.2mm lateral; -1.3mm ventral from surface of brain). For viral tracing experiments 100nl of CTb-Alexafluor594 (Abcam) or pAAV-Syn-ChR2(H134R)-GFP (AAV Retrograde; Addgene) was injected bilaterally into the LH. To chronically ablate the ACC to LH circuit CAV2-cre-GFP (Montpeiler Vector Core, France) injected bilaterally into the LH at a volume of 200nl and the cre-dependent caspase virus (AAV-flex-taCasp3-TEVp; UNC vector core, gifted by Nirao Shah & Jim Wells or AAV-GFP; Addgene for controls) was injected bilaterally into the ACC at a volume of 300nl. To insert the stimulatory DREADDs into the ACC to LH projection neurons AAV-pgk-cre (retrograde; Addgene) was injected bilaterally into the LH at a volume of 100nl before the cre-dependent viral construct encoding for the stimulatory DREADDs receptor (pAAV5-hSyn-DIO-hM3D(Gq)-mCherry; Addgene or AAV-GFP; addgene for controls) was injected bilaterally into the ACC at a volume of 300nl.

Immunohistochemistry

After experiments, mice were deeply anesthetised and then transcardially perfused with 0.05M phosphate buffered (PB) saline followed by 4% *w/v* paraformaldehyde (PFA) in 0.1 M PB. Brains were post-fixed in 4% PFA in 0.1 M PB overnight before being transferred to 30% sucrose solutions. Coronal sections of the whole brain were cut at 30 μm in sets of four. A one in four series of tissue sections was washed in 0.1 M PB three times before being blocked in 1% H_2O_2 in 0.1 M PB for 15 min and then washed again. Tissue was then placed in 0.3% Triton X-100 in 0.1 M PB and 4% normal horse serum for one hour before an overnight incubation at 4°C in primary antibody: anti-NeuN (rabbit, 1:1000, Abcam), anti-mcherry (chicken, 1:1000, Abcam). Tissue sections were then washed again before a 90 min incubation in secondary antibody at room temperature; goat anti-rabbit IgG AlexaFluor 488 (1:400; Life Technologies), goat anti-chicken IgG AlexaFlour 594 (1:400, Life Technologies), Alexa fluor goat-antirabbit IgG (1:400, Life technologies). The

sections were then mounted and coverslipped with hard set mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) (Vector) used to counterstain DNA. To confirm cell ablation in caspase experiments images of NeuN stained tissue were analysed using Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.) to determine % area of cell coverage.

DREADDs experiments

For activation of DREADDs all mice (control [AAV-GFP] and experimental [AAV-DIO-hM3DGq-mCherry]) were injected in the intraperitoneal cavity with CNO (1mg/kg; Sigma) 30 minutes to one hour before each experiment commenced.

Behaviour

Behavioural analysis

All behavioural analysis was performed blinded to treatment using (Ethovision version 14.0.1322, Noldus Information Technology).

Elevated plus maze

The elevated plus maze (400 mm above floor) consisted of two open arms (310 mm X 50 mm) and two closed arms (310 mm X 50 mm with 150 mm high barriers). Mice were placed in the centre of the plus maze facing an open arm and allowed to explore the apparatus for the duration of the test (6 minutes). The time spent exploring both open arms was measured. The time spent in the centre of the plus maze was excluded.

Light dark box

The light-dark box (285 mm wall height) was constructed from wood and consisted of a larger light chamber (480 mm X 295 mm; painted white) and a smaller dark chamber (150 mm X 295 mm; painted black) separated by a small opening. Mice were placed in the centre of the dark chamber and allowed to move freely about the two chambers for the duration of the test (6 minutes). The time the mouse spent in the light chamber was measured.

Open field

Mice were placed in the outer ring of a large open field (800 mm diameter with wall height 300 mm) and allowed to freely move about the apparatus for the duration of the test (6 minutes). Time spent in the centre zone (200 mm diameter) was measured.

Baited behavioural tests

Baited behavioural tests have previously been optimised in our lab (Lockie et al., 2017). Mice were exposed to peanut butter chips (Reeces, USA) randomly three times during the week leading up to the baited behavioural tests. Mice either had *ad libitum* access to chow, or were fasted overnight (15 hours). One peanut butter chip was weighed and then placed in the centre of the light chamber of the light-dark box described above. Mice were placed in the dark zone and then given six minutes to freely explore the apparatus. The time spent in the food zone (50mm radius around the pellet), the amount of the pellet consumed, entries into the food zone and entries into the dark zone were measured. On a separate day mice were placed into the open field, which was baited with three peanut butter chips, placed in the centre region. Mice were placed in

the outer ring of the open field and then given six minutes to move about the apparatus. The time spent in the food zones (50mm radius around pellets), amount of pellet consumed, entries into food zone and entries into the outer zone were measured. All mice completed each baited test in both the fed and fasted states separated by one week. Groups were counter balanced to the order of treatment.

Food and liquid intake

Home cage food intake

For chronic circuit ablation caspase experiments, fasting re-feeding during the light phase food was removed two hours before the onset of the dark phase and returned two hours after the beginning of the light phase. For DREADDs feeding during the dark phase experiment food was removed one hour before dark phase onset and returned 3 hours later, CNO (1mg/kg; Sigma) was injected 30 minutes before food was returned and intake was measured at two hours and 4 hours (Fig 5B).

Saccharin and Sucrose Preference Tests

Mice were placed in feeding cages (BioDaq) and given *ad lib* access to standard chow and water for 4 days during the acclimation period. During the saccharin preference test mice had access to water on one side of the cage and saccharin (0.1% *w/v*, Sigma) on the other. Sides of the bottles were reversed after two days. Total intake of saccharin and water was recorded for 4-5 days. Before beginning the sucrose preference test, there was a 2-day washout period where mice only had access to water and chow. During the sucrose preference test mice had access to saccharin (0.1% *w/v*, Sigma) on one side of the cage and sucrose (4% *w/v*, Colonial Sugar Refining Company) on the other. Water was not available during this period. These concentrations were chosen as they had previously been shown to be of similar sweetness and are iso-prefered by mice (Bachmanov et al., 2001). Mice had 3 days access to sucrose and saccharin. On the 3rd night food was removed from the cage and intake of sucrose and saccharin were recorded over the fasting period.

High Fat Diet Preference

Following the sucrose preference test in the feeding cages and a second washout period, mice were given *ad libitum* access to chow in one food hopper, and high fat/high sugar chow (23% fat, Specialty Feeds) in the other. Food intake was recorded for 4 days. On the 4th night access to the food hoppers was blocked at 5pm before being opened again at 9am the following day. Food intake in response to the overnight fast was recorded for 24 hours.

Statistical Analysis

Data are expressed mean with standard error of the mean. Comparisons were tested using two-tailed unpaired t-tests or paired t-tests. A repeated measures (where appropriate), two-way ANOVA with Sidak's multiple comparison test was applied when there both fasting and fed conditions, or when a variable was measured over time. $p<0.05$ was considered statistically significant. Graphs were generated in Prism (Version 8.4, 2020).

4.3 Results

Chronic circuit ablation

Initially, we confirmed the presence of dense monosynaptic ACC-LH projections in mice using the retrograde tracer Cholera toxin B (CTb) bilaterally injected into the LH (Fig 1A). Significant uptake of the red fluorophore is observed throughout mouse ACC which is comprised of the cingulate gyrus regions 1 (Cg1) and 2 (Cg2) (Fig 1B). Based on the results of these tracing studies we defined our ACC target region as Cg2 as this where the greatest density of projection neurons were observed, a region that is anatomical equivalent to the ACC in humans(van Heukelum et al., 2020). To chronically ablate the ACC-LH circuit we used a dual viral approach (Fig 1D). Cav2-cre was used to insert cre-recombinase into ACC-LH projection neurons and a genetically engineered cre-dependent caspase virus was then targeted to these neurons (AAV-flex-tacasp3-TEVp), which results in caspase-mediated apoptosis as described in Yang *et al* 2013 (Yang et al., 2013). We confirmed cell ablation by staining for the neuronal marker NeuN and determining % area of cells in the ACC that were immunoreactive for NeuN (Fig 1E). Caspase expression significantly reduces % NeuN area in the ACC-LH group by approximately 5% (Fig 1E). Chronic circuit ablation had no effect on % body weight gain on 14-week chow feeding period (1F). However, circuit ablation increases the amount of chow consumed over a 4-day *ad lib* period in BioDaq feeding cages (Fig I-J). Despite this, when challenged with an overnight fast, mice in the circuit ablation group consume the same as controls suggesting the ACC-LH circuit is not required for the normal homeostatic response to energy deficit (Fig 1G-H). To determine whether this circuit could influence palatable food intake, mice were placed on an 8-week *ad lib* high fat/high sugar diet. Chronic circuit ablation increased % body weight gain (Fig 1K) and average daily high fat/high sugar diet intake (Fig 1L-M) over a 4-day *ad lib* feeding period in the BioDaq feeding cages. During this period in the feeding cages mice also had access to chow, however all mice consumed only the high fat/high sugar diet. Our results from the chow feeding period suggest the ACC-LH circuit influences normal feeding patterns, but is not involved in the homeostatic drive to eat. To confirm whether this is still the case when mice have access to a palatable diet, we fasted mice overnight and measured high fat/high sugar intake over the following 24 hour period. Consistent with the chow feeding period, when presented with a homeostatic challenge mice in the circuit ablation group consume a similar amount as controls (Fig 1N), with any trend observed here a result of differences in bodyweight (1O). While there were no differences in total amount of palatable diet consumed between groups, there is an interaction between circuit ablation and cumulative high fat/ high sugar diet over time (Fig 1P), indicating that the ACC-LH may influence the rate at which the energy deficit is restored across the light and dark phases. Together these results suggest the ACC-LH circuit may function as an anorexigenic circuit, in that ablation increases chow intake, increases palatable diet intake and increases % body weight gain on a palatable diet. However, results from fasting re-feeding experiments indicate the ACC-LH circuit is not required for mediating the response to a homeostatic challenge.

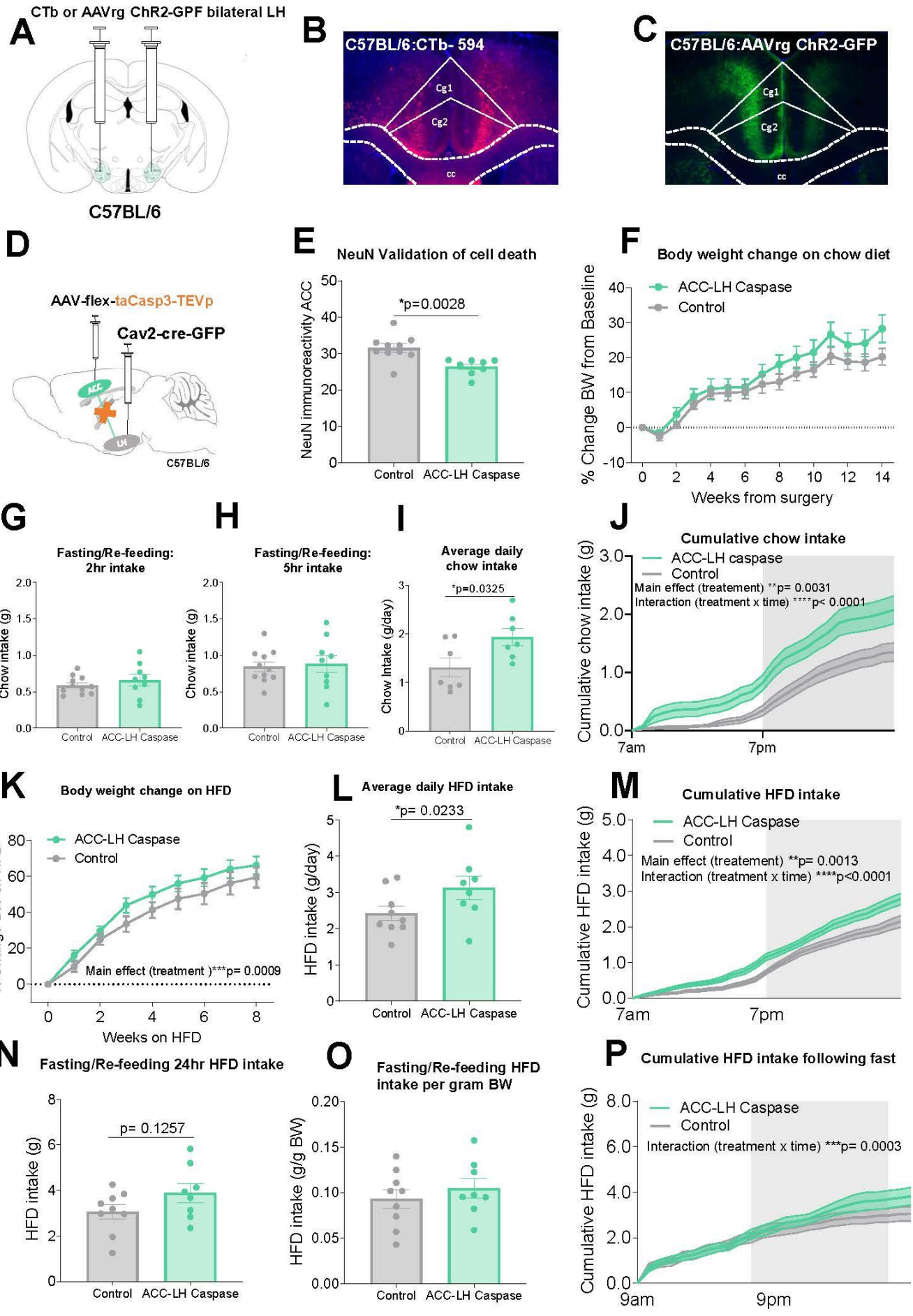

A
CTb or AAVrg ChR2-GPF bilateral LH
C57BL/6
B
C57BL/6:CTb- 594
Cg1
Cg2
cc
C
C57BL/6:AAVrg ChR2-GFP
Cg1
Cg2
cc
D
AAV-flex-taCasp3-TEVp
Cav2-cre-GFP
ACC
LH
C57BL/6
E
NeuN Validation of cell death
NeuN immunoreactivity ACC
*p=0.0028
Control
ACC-LH Caspase
F
Body weight change on chow diet
% Change BW from Baseline
ACC-LH Caspase
Control
Weeks from surgery
G
Fasting/Re-feeding:
2hr intake
Chow intake (g)
Control
ACC-LH Caspase
H
Fasting/Re-feeding:
5hr intake
Chow intake (g)
Control
ACC-LH Caspase
I
Average daily
chow intake
*p=0.0325
Chow Intake (g/day)
Control
ACC-LH Caspase
J
Cumulative chow intake
ACC-LH caspase
Control
Main effect (treatement) **p= 0.0031
Interaction (treatment x time) ****p< 0.0001
Cumulative chow intake (g)
7am
7pm
K
Body weight change on HFD
ACC-LH Caspase
Control
Main effect (treatment)***p= 0.0009
% Change BW on HFD
Weeks on HFD
L
Average daily HFD intake
*p= 0.0233
HFD intake (g/day)
Control
ACC-LH Caspase
M
Cumulative HFD intake
ACC-LH Caspase
Control
Main effect (treatement) **p= 0.0013
Interaction (treatment x time) ****p<0.0001
Cumulative HFD intake (g)
7am
7pm
N
Fasting/Re-feeding 24hr HFD intake
p= 0.1257
HFD intake (g)
Control
ACC-LH Caspase
O
Fasting/Re-feeding HFD
intake per gram BW
HFD intake (g/g BW)
Control
ACC-LH Caspase
P
Cumulative HFD intake following fast
ACC-LH Caspase
Control
Interaction (treatment x time) ***p= 0.0003
Cumulative HFD intake (g)
9am
9pm

Figure 1. Chronic ablation of ACC-LH circuit results in increased chow consumption, high fat/high sugar diet consumption and increased bodyweight gain on high fat/high sugar diet. The retrograde tracer Cholera-toxin B (CTb) conjugated with Alexa fluorophore 594 or Retrograde AAV ChR2-GPF was injected bilaterally into the LH of C57BL/6 mice (A). A representative image shows the transport of the red fluorophore throughout the mouse ACC, which is comprised of the cingulate gyrus 1 (Cg1) and cingulate gyrus 2 (Cg2) regions (B). A representative image shows expression of the reporter protein GFP throughout Cg1 and Cg2 (C). The retrograde canine adenovirus-2 encoding for Cre-recombinase (CAV2-cre-GFP) was injected bilaterally into the lateral hypothalamus of C57BL/6 mice before a cre-dependent AAV construct encoding for a genetically engineered caspase-3 was injected bilaterally into the ACC in the Cg2 region as this is where the highest density projection neurons were observed (D). Caspase injection reduced % area of immunoreactivity of the neuronal marker NeuN in the ACC (E; n=8-10, Student's unpaired t-test). Circuit ablation did not affect % body weight gain on a chow diet (F). Circuit ablation did not affect chow intake at 2 hours (G) or 5 hours (H) following an overnight fast. There was a main effect of circuit ablation on cumulative chow intake and an interaction between circuit ablation and cumulative chow intake over time (I; n=7, Student's unpaired t-test). Circuit ablation did not affect cumulative chow intake over the 4-day period in the BioDaq feeding cages (J). Main effect of circuit ablation on % body weight gain on a high fat/high sugar diet (K; 9-11, two-way ANOVA with Sidak's multiple comparison test). Circuit ablation increased average daily high fat/high sugar diet over a 4-day period in the BioDaq feeding cages (L; n=9, Student's unpaired t-test). There was a main effect of circuit ablation on cumulative high fat/high sugar diet intake and an interaction between circuit ablation and cumulative high fat/high sugar intake over time (M; n=9, two-way ANOVA with Sidak's multiple comparison test). Circuit ablation had no effect on total high fat/high sugar diet intake or high fat/high sugar diet intake per gram body weight following an overnight fast (N&O). There was an interaction between circuit ablation and cumulative high fat/high sugar diet intake over time following an overnight fast (P; n=8-9, two-way ANOVA with Sidak's multiple comparison test). Data are presented mean ± SEM. ACC – anterior cingulate cortex; BW – bodyweight; cc – Corpus callosum; Cg – Cingulate gyrus; HFD – high fat/high sugar diet. Grey shading in J,M & P indicates dark phase.

Previous studies into the function of genetically defined populations or projections of LH neurons demonstrate a role for the LH in anxiety-like behaviour and locomotor activity.(Cassidy et al., 2019; Patterson et al., 2015; Qualls-Creekmore et al., 2017). To determine whether the ACC-LH circuit might influence anxiety-like behaviour or locomotor activity, we tested mice in the elevated plus maze (EPM) (Fig 2A) and open field (Fig 2D). Chronic circuit ablation has no effect on %time spent in open arms (Fig 2B) or entries into the open arms (Fig 2C). Similarly, in the open field there is no effect of circuit ablation on %time spent in the inner zone (Fig 2E) or entries into the outer zone from the inner zone (Fig 2F). Together with the results from the EPM, we conclude that chronic ACC-LH circuit ablation does not influence anxiety like behaviour. Velocity (Fig 2G) and distance travelled (Fig 2H) in the open field were also not different between groups indicating that chronic ACC-LH circuit ablation does not affect locomotor activity.

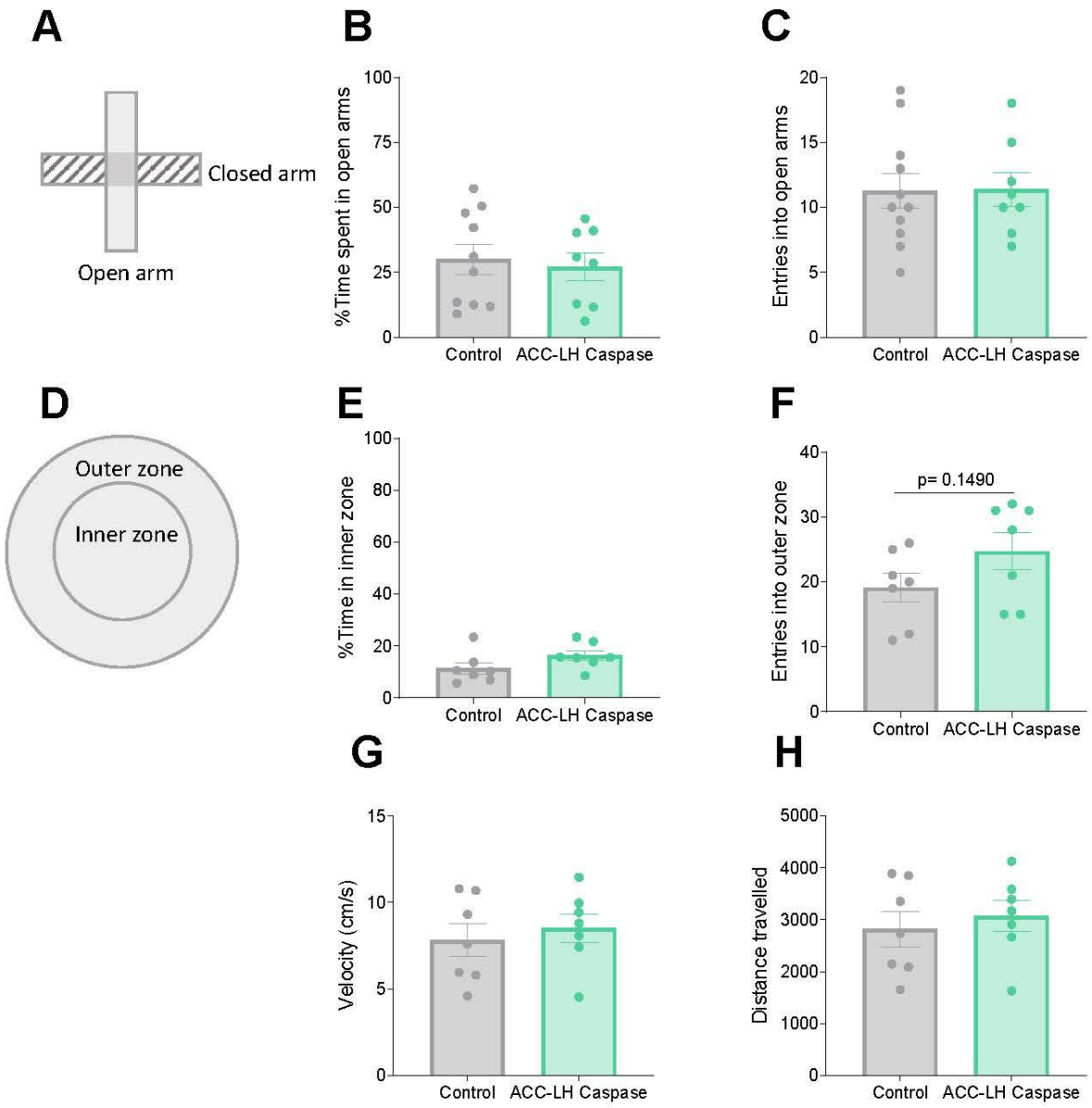


Figure 2. Chronic ablation of ACC-LH circuit has no effect on anxiety-like behaviour or locomotor activity. Elevated plus maze apparatus (A). Chronic circuit ablation had no effect on %time spent in open arms (B) or entries into open arms (C). Open field apparatus (D). Chronic circuit ablation had no effect on %time spent in the inner zone (E), entries into the inner zone (F), velocity (G) or distance travelled (H) in the open field. Data are presented mean ± SEM.

Perturbations to ACC activity in humans have been implicated in altered valuation of reward and aversion to risk, ultimately increasing the likelihood an individual would engage in risk taking behaviour to obtain a reward (Alexander et al., 2015). To test whether ablation of the ACC-LH circuit would increase risk taking behaviour to obtain a palatable food reward we used baited anxiogenic environments (Fig 3A&G)(Lockie et al., 2017). ACC-LH circuit ablation has no effect on the amount of peanut butter pellet (bait) consumed (Fig 3B) or any behavioural variable assessed in the baited light-dark box (Fig 3B-F). As expected, fasted mice consume more of the peanut butter pellet (Fig 3B) and spend more time in the risky food zone (Fig 3A-B), illustrating the effect hunger has on behaviour. Circuit ablation also does not affect peanut butter pellet intake in the baited open field (Fig 3H) or any behaviour assessed during the test (Fig 3I-L). Again, fasting increases pellet consumption (Fig 3H), time in the risky food zone and also entries into the food zone and time in the inner

zone (Fig 3I-K) in the baited open field. Together these results suggest ACC-LH circuit ablation does not influence palatable food seeking in a risky environment in either the fed or fasted state. The response to fasting in these experiments is similar between groups and therefore supports earlier fasting re-feeding experiments that demonstrate the ACC-LH circuit ablation does not affect the homeostatic drive to eat. From these experiments, we conclude the ACC-LH circuit is not required to maintain either the appropriate behavioural or feeding response to acute homeostatic challenges.

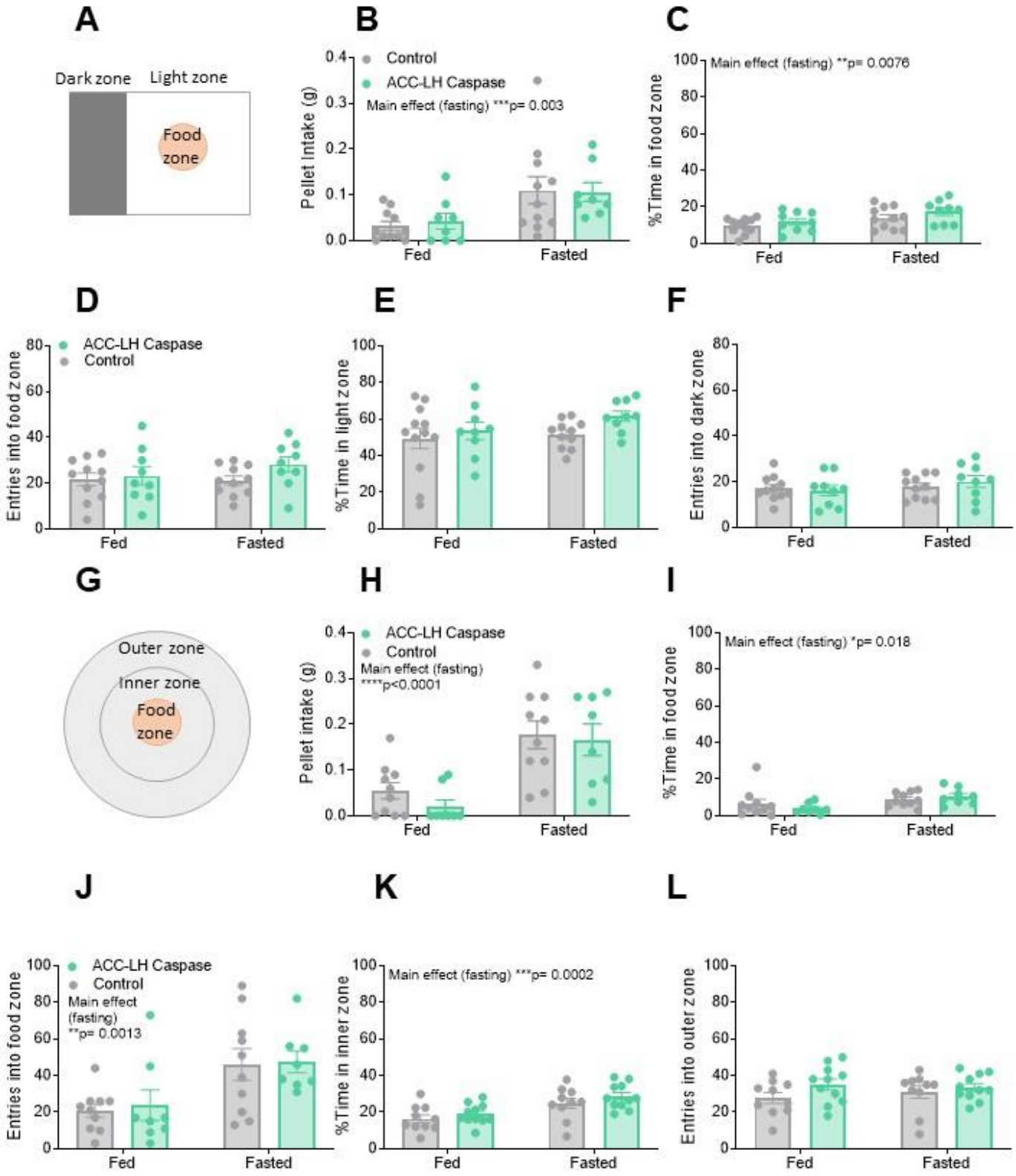


Figure 3. Chronic ablation of ACC-LH circuit has no effect on risk-reward behaviour as assessed by baited behavioural tasks. Baited light-dark box: One peanut butter pellet was placed in the centre of the light zone as indicated by the food zone (A). Chronic circuit ablation had no effect on pellet intake or %time spent in food zone, however there was a main effect of metabolic state (fed vs fasted; B-C; n=9-11, two-way ANOVA with Sidak's multiple comparison test). Chronic circuit ablation and metabolic state had no effect on entries into food zone (D), %time in light zone (E), or entries into the dark zone from the light zone (F). Baited open field: 3 peanut butter pellets were placed in the centre of the inner zone as indicated by the food zone (G). Chronic circuit ablation had no effect on pellet intake, %time spent in food zone, entries into food zone or %time in inner zone, however there was main effect of metabolic state (fed vs fasted; H-K; n=9-11, two-way ANOVA with Sidak's multiple comparison test). Chronic circuit ablation and metabolic state had no effect on entries into outer zone from inner zone (L). Data are presented mean ± SEM.

The LH is a key brain region involved in the expression of motivated reward seeking behaviours, including compulsive sucrose seeking (Nieh et al., 2015). The ACC is also broadly implicated in reward valuation as neurons in this regions modify their firing rates in proportion to the value of obtained reward (Heilbronner & Hayden, 2016). This led us to further investigate any role for the ACC-LH in reward processing through a series of two bottle preference tests comparing preference for sweet non-nutritive (saccharin) and sweet energy containing (sucrose) solutions (Fig 4). Firstly, mice were given access to a saccharin solution and water (Fig 4A). Circuit ablation does not significantly affect average daily saccharin solution consumption (Fig 4C) or preference for saccharin solution over water (Fig 4B&D). However, there is a main effect of circuit ablation on cumulative saccharin intake over the light-dark cycle and an interaction between circuit ablation and the rate of saccharin intake over the light-cycle. These results indicate that ACC-LH circuit ablation results in a mildly anhedonic phenotype in this context, and also influences consumption of a rewarding liquid solution differentially across the light-dark cycle. We next asked whether circuit ablation also affects intake of caloric versus non-calorie rewards since reward value assigned to sweet taste and calorie content are mediated through separate neural circuits (Tellez et al., 2016). To investigate any differences in processing of hedonic versus nutritive rewards associated with circuit ablation, we gave mice access to a saccharin solution and a sucrose solution in both fed and fasted states, without water available (Fig 4F&K). There is a tendency for circuit ablation to reduce average daily saccharin intake (Fig G; p=0.0777) and sucrose intake (Fig 4H; p=0.0623) in a fed state, though this does not reach significance, and does not alter preference for sucrose over saccharin (Fig 4I), which remains above 50% for both groups. There is a main effect of ACC-LH circuit ablation on cumulative sucrose intake over time, and an interaction between circuit ablation and the rate of saccharin intake over the light-cycle (Fig 4J). These results support those of the previous saccharin vs water preference test and indicate that the ACC-LH circuit is required for normal consumption of sweet tasting (and therefore rewarding) solutions, and differentially affects intake across the light-dark cycle. Given our feeding experiments show that the ACC-LH circuit ablation does not affect chow or high fat/high sugar consumption in response to a fast, we next tested whether circuit ablation would influence liquid sucrose consumption when mice were deprived of food (Fig 4K). As expected, when mice are fasted overnight (food removed but bottles available) sucrose consumption is dramatically increased (Fig 4Q) confirming the appropriate detection of calorie need. ACC-LH circuit ablation does not affect average daily sucrose consumption (Fig 4M), energy intake (Fig 4O) or sucrose preference over saccharin (Fig 4L&N) during an overnight fast. However, there is an interaction between circuit ablation and the rate at which energy

requirements are met through sucrose consumption (Fig 4P&Q) –although the difference here may reflect the fact that control mice were in energy surplus before entering the fast due to higher sucrose consumption in the days prior (Fig 4H). These results help to confirm that chronic ACC-LH circuit ablation does not influence consummatory behaviour in response to a homeostatic challenge. From this set of experiments, we conclude that ACC-LH circuit ablation results in a mildly anhedonic phenotype, where consumption of both caloric and non-caloric rewards appear to be reduced in a manner that appears to be related to stage of the light-dark cycle.

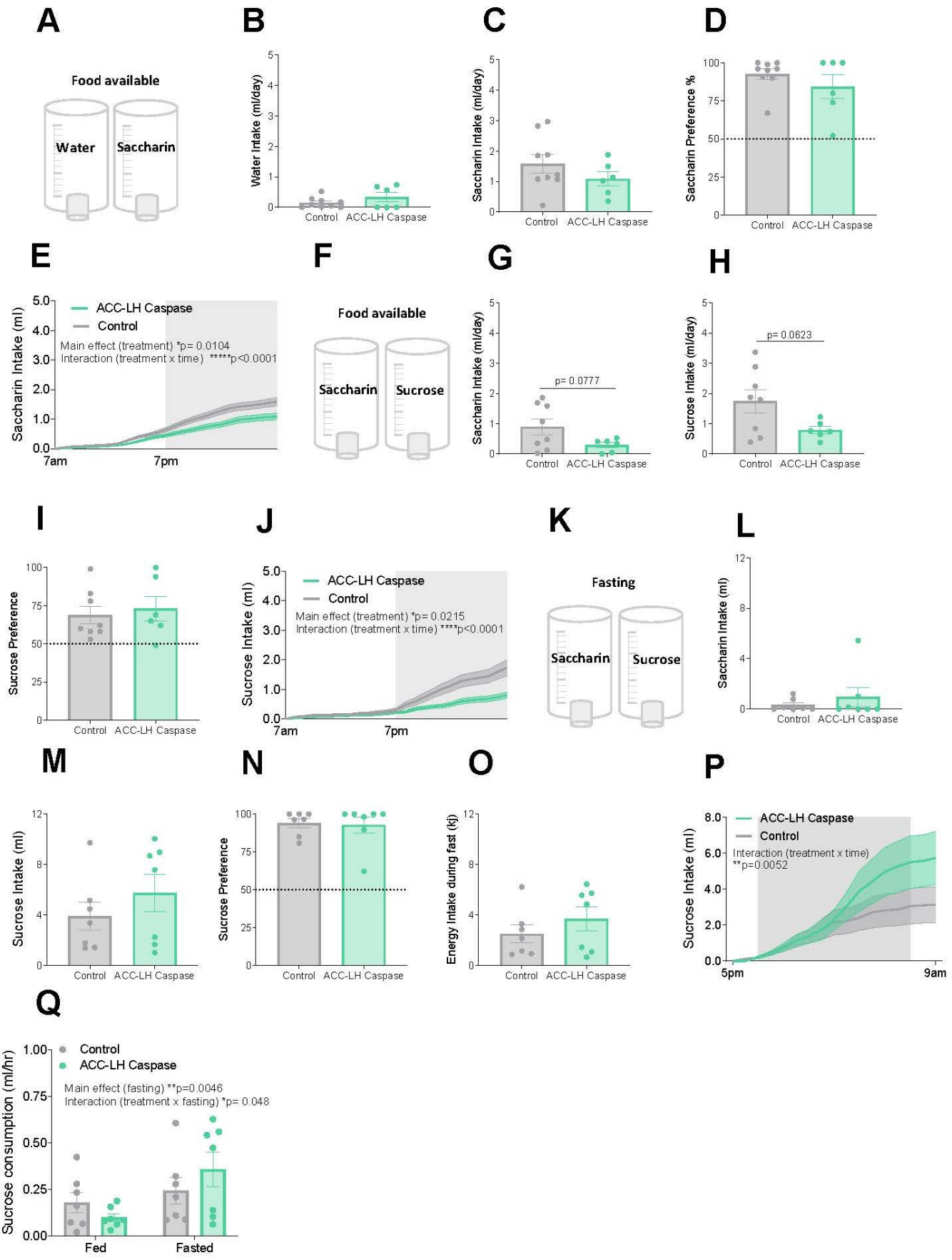


Figure 4. Chronic ablation of ACC-LH circuit reduces cumulative saccharin and sucrose intake over time in a two bottle preference test but does not affect sucrose intake during fasting. Mice were given *ad lib* access to bottles containing water and saccharin and *ad lib* access to food for 4 days (A). Chronic

circuit ablation had no effect on water intake, saccharin intake, or preference for saccharin (B-D). There was a main effect of circuit ablation on cumulative saccharin consumption over time and an interaction between circuit ablation and cumulative saccharin consumption over time (E; n=6-8, two-way ANOVA with Sidak's multiple comparison test). Mice were given *ad lib* access to bottles containing saccharin and sucrose with *ad lib* access to chow for 4 days (F). Chronic circuit ablation had no significant effect on saccharin intake, sucrose intake, or preference for sucrose, though there was a strong trend for reduced saccharin and sucrose consumption in the circuit ablation group (G-I). There was a main effect of circuit ablation on cumulative sucrose intake and an interaction between circuit ablation and cumulative sucrose consumption over time (J; n=6-8, two-way ANOVA with Sidak's multiple comparison test). Mice were given *ad lib* access to bottles containing saccharin and sucrose during an overnight fast (K). Chronic circuit ablation had no effect on saccharin intake, sucrose intake, or energy intake during fasting (L-0). There was an interaction between circuit ablation and time for cumulative sucrose intake during an overnight fast (P; n=7, two-way ANOVA with Sidak's multiple comparison test). There was no effect of circuit ablation on sucrose intake per hour, although a main effect of metabolic state (fed vs fasted) and an interaction between circuit ablation and metabolic state was observed (Q; n= 8-10, two-way ANOVA with Sidak's multiple comparison test). Data are presented mean ± SEM. Grey shading in E,J & P indicates dark phase.

Acute circuit activation with DREADDs

Our results from the chronic circuit ablation studies suggest the ACC-LH circuit may have an anorexigenic role, since deletion of this circuit resulted in increased consumption of a both chow and palatable diets, and increased body weight gain on a palatable diet. We therefore predicted that acutely activating the ACC-LH circuit would inhibit feeding. To activate this circuit, we used a dual viral approach to express the stimulatory DREADD hM3Dq into ACC-LH projecting neurons (Fig 5A). Firstly, to determine whether acute ACC-LH activation inhibits feeding, we examined food intake following a short term fast during the dark phase the period when mice consume the majority of their energy intake (Fig 5B). Acute activation of the ACC-LH circuit suppresses food intake at 2 hours (5C) and 4 hours (5D) after food was available during the dark phase. This supports the hypothesis that the ACC-LH circuit is an anorexigenic circuit.

To determine whether there are any anxiety behaviours associated with activation of the ACC-LH circuit we used the EPM light dark box (Fig 5E-M). Acute circuit activation did not affect % time in the open arms in the EPM (Fig 5E) or % time in the light zone of the light-dark box (Fig 5H), but did increase the number of entries into the open arms (Fig 5F) and number of entries into the light-zone (Fig 5I). We then considered whether this was a result of an increase in exploratory behaviour or due to an increase in locomotor activity in general. Acute circuit activation increases distance travelled in both the EPM (Fig 5G) and light-dark box (Fig 5J) suggesting the increased entries to the anxiogenic zones are likely a result of increased locomotor activity. To examine this further, mice were tested in the open field apparatus (Fig 5K-M). Although % time in the aversive inner zone was not affected (Fig 5K), acute ACC-LH circuit activation dramatically increased velocity (Fig 5L) and distance travelled in the open field (Fig 5M). The results from the DREADDs studies suggest the ACC-LH circuit is anorexigenic in nature, and promotes increased locomotor activity in behavioural arenas.

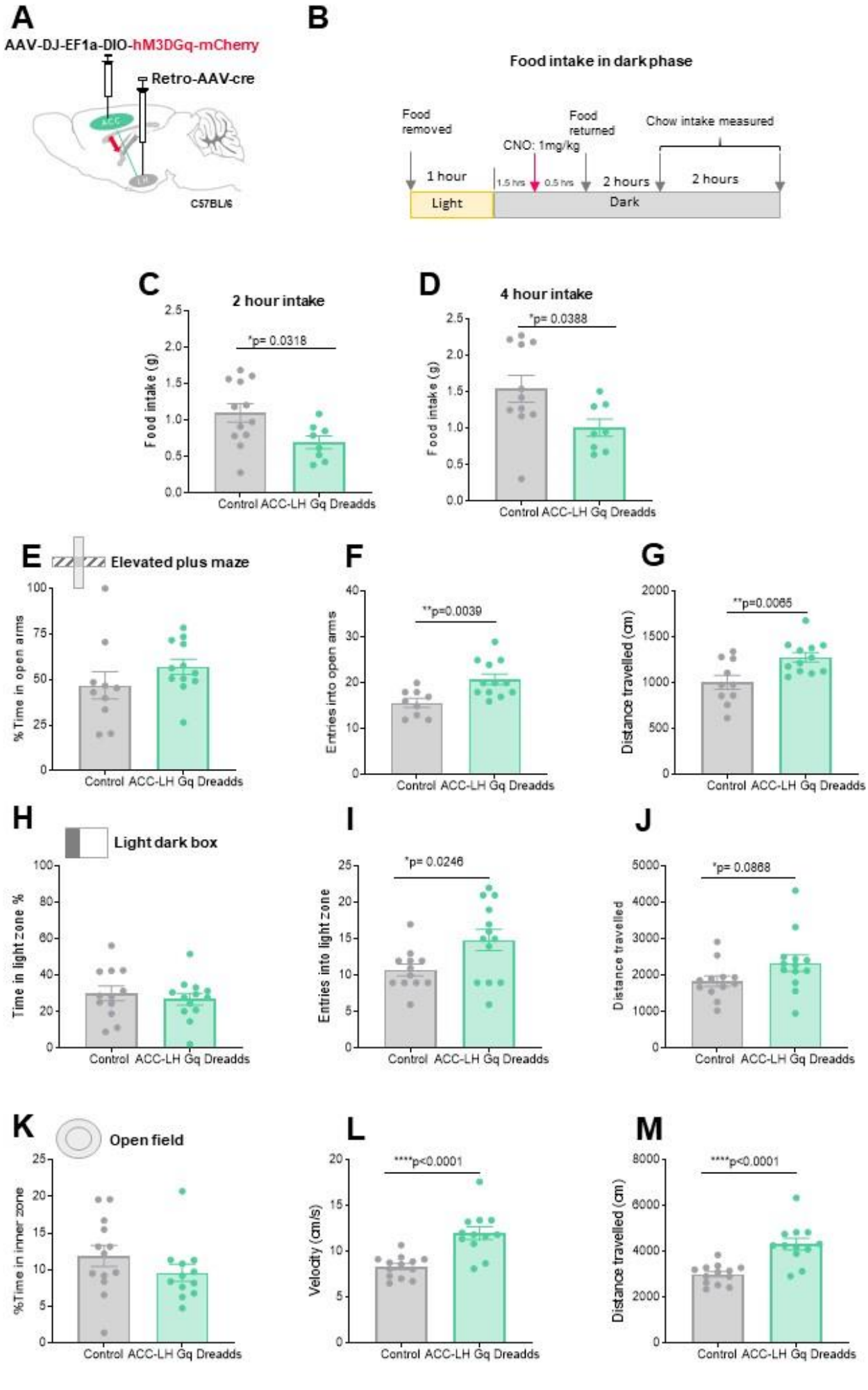

A
AAV-DJ-EF1a-DIO-hM3DGq-mCherry
Retro-AAV-cre
ACC
LH
C57BL/6
B
Food intake in dark phase
Food removed
Food returned
Chow intake measured
CNO: 1mg/kg
1 hour
1.5 hrs
0.5 hrs
2 hours
2 hours
Light
Dark
C
2 hour intake
*p= 0.0318
Food intake (g)
Control ACC-LH Gq Dreadds
D
4 hour intake
*p= 0.0388
Food intake (g)
Control ACC-LH Gq Dreadds
E
Elevated plus maze
% Time in open arms
Control ACC-LH Gq Dreadds
F
**p=0.0039
Entries into open arms
Control ACC-LH Gq Dreadds
G
**p=0.0065
Distance travelled (cm)
Control ACC-LH Gq Dreadds
H
Light dark box
Time in light zone %
Control ACC-LH Gq Dreadds
I
*p= 0.0246
Entries into light zone
Control ACC-LH Gq Dreadds
J
*p= 0.0868
Distance travelled
Control ACC-LH Gq Dreadds
K
Open field
%Time in inner zone
Control ACC-LH Gq Dreadds
L
****p<0.0001
Velocity (cm/s)
Control ACC-LH Gq Dreadds
M
****p<0.0001
Distance travelled (cm)
Control ACC-LH Gq Dreadds

Figure 5. Acute activation of ACC-LH circuit using Gq DREADDs suppresses food intake and increases locomotor activity. A retrograde AAV virus encoding for cre-recombinase was injected bilaterally into the LH of C57BL/6 mice before a cre-dependent AAV encoding for the human muscarinic receptor hM3Dq and m-Cherry reporter protein was injected bilaterally into the ACC (A). Experimental timeline used to assess food intake in the dark phase following an acute fast and ACC-LH circuit activation with CNO (B). Circuit activation suppressed food intake at 2 hours and 4 hours (C-D; n=8-12, Student's unpaired t-test) following an acute fast in the dark phase. Acute circuit activation had no effect on %time spent in open arms in the elevated plus maze (E), but increased entries into the open arms and distance travelled in the elevated plus maze (F-G; n=11-12, Student's unpaired t-test). Acute circuit activation had no effect on %time spent in inner zone in the light-dark box (H) but increased entries into the light zone and distance travelled in the light dark box (I-J; n=12-13, Student's unpaired t-test). Acute circuit activation had no effect on %time spent in inner zone in the open field (K) but increased velocity and distance travelled (L-M; n=12-13, Student's unpaired t-test).Data are presented mean ± SEM. Cg – cingulate gyrus; 1 CNO –Clozapine-N-oxide.

4.4 Discussion

The ACC is implicated in a diverse range of functions, however the control of reward-based decision-making is the most commonly reported observation (Shenhav et al., 2016). Although there has been considerable research into this brain region in both humans and animals – precisely how the ACC acts to influence broad reward-related behaviour is not clear. The limited number of animal studies investigating the role of the ACC specifically in the valuation of food have produced conflicting, and often confusing results. Some studies report disruption of normal neuronal activity in the ACC, either through chemogenetic activation or inhibition (Hart et al., 2019), or through pharmalogical lesions (Hart et al. 2017) suppresses motivation to obtain a sucrose reward, but only when there is a choice to consume freely available, but lower reward-value, standard rodent chow. In contrast, other studies show that inactivation of the ACC through pharmacological lesions (Zhong et al., 2017) promotes foraging for highly palatable food over social interaction, which is preferred in control animals. Discrepancies in these studies could be explained by heterogeneous activity patterns of the neurons targeted as there is evidence of sub-populations of ACC neurons that display opposing activity profiles during goal-directed tasks (Hart et al., 2019; Kvitsiani et al., 2013). In addition, the differences in the studies described could arise from heterogeneous projection sites of the targeted ACC neurons. There is evidence to suggest that within other frontal cortices, such as the mPFC, functional output of neuronal activity might be specific to downstream targets (Otis et al., 2017b; Verharen et al., 2020). In this study, our results reveal a role for the ACC-LH circuit in the overconsumption of both low-value and high-value foods and ultimately indicate that this circuit is anorectic. Chronic ablation of the ACC-LH circuit increases food intake and bodyweight on a high fat/high sugar diet, while activation of the ACC-LH circuit suppresses food intake in the dark phase and increases locomotor activity in behavioural arenas. Our results fit with recent studies showing that direct cortical to LH circuits provide top down control over the homeostatic drive to eat (Wu et al., 2020).

Within the LH, GABAergic neurons appear to drive feeding and promote appetitive behaviours, while *Vglut2* expressing neurons suppress feeding and are aversive (Jennings et al., 2013, 2015; Stamatakis et al., 2016). However, studies that investigated photostimulation or genetic ablation of LH^{Vglut2} do not report any changes to locomotor activity (Jennings et al., 2013; Stamatakis et al., 2016) – suggesting that the increased activity we observe in acute ACC-LH activation is not a result of ACC inputs onto LH^{Vglut2} neurons. The LH also contains a large population of neurons that express the anorectic neuropeptide Neurostensin (Nts) (Schroeder & Leinninger, 2018), and these are distinct from LH neurons that express melanin concentrating hormone (MCH) and orexin (Laque et al., 2013; Stuber & Wise, 2016). Some LH^{Nts} coexpress vesicular GABA transporter (*Vgat*) (Jennings et al., 2015; Patterson et al., 2015), however do not promote food intake when activated, as seen with broad LH^{Vgat} activation (Jennings et al., 2013, 2015; Nieh et al., 2015). Approximately, 15-30% of LH^{Nts} neurons coexpress the leptin receptor (LepRb) and mediate the anorectic actions of leptin and are therefore required to maintain energy balance (Leinninger et al., 2011). Chemogentetic activation of LH^{Nts} neurons increases locomotor activity and suppresses chow intake (Woodworth, Beekly, et al., 2017) in a similar manner to the results we see following acute activation of the ACC-LH circuit, therefore suggesting that ACC neurons that project to the LH may target LH^{Nts} neurons. In this study, acute activation of LH^{Nts}

neurons also reduces operant responding for sucrose pellets (Woodworth, Beekly, et al., 2017). We are planning to test the effect of acute ACC-LH circuit activation on motivated sucrose seeking behaviour in order to determine if further results are consistent with ACC modulation of LH^{Nts} neurons. However, one limitation of this study is that we have not determined if the ACC to LH neurons also project to other regions. The ACC has dense connections with the motor cortex and spinal cord (Paus, 2001), which could be relevant to the increase in locomotor activity we have observed. To overcome this, we plan to activate nerve terminals of ACC neurons in the LH using optogenetics.

While we observed consistent increases in locomotor activity with acute ACC-LH circuit activation, chronic circuit ablation did not affect locomotor activity in any of the behavioural tasks examined. It is possible that chronic circuit ablation may result in compensation of any deficits to locomotor activity through increased activity of other neural circuits. Secondly, locomotor activity might be more sensitive to acute manipulations of the ACC-LH circuit rather than sustained changes to signalling. In support of this idea, there have been discrepancies in the findings related to locomotor activity in studies manipulating LH^{GABA} neurons: acute chemogenetic activation of LH^{GABA} neurons reduces locomotor activity (de Vrind et al., 2019); while chronic ablation of LH^{GABA} neurons has no effect on locomotor activity (Jennings et al., 2015). Finally, our chronic circuit ablation studies were performed during the light-phase, while acute activation studies were performed in the dark-phase, suggesting differences between the two models could be a result of interactions with the ACC-LH circuit and circadian rhythms.

Chronic ACC-LH circuit ablation resulted in a mild anhedonia during access to sweet-solutions in the feeding cages when mice had access to chow. This disappeared during the overnight fast demonstrating that the ACC-LH circuit functions independently from homeostatic circuits. The reduction in sucrose intake during the free-feeding period seems inconsistent with our observations that chronic ACC-LH circuit ablation increases consumption of a high fat/high sugar diet, and that acute ACC-LH circuit activation suppresses re-feeding following a short-term fast. Possibly, the ACC-LH circuit senses the rewarding properties of a liquid sucrose solution differently to those of a whole food that has both high carbohydrate and high fat content. We aim to test this idea by giving access to sweet-solutions and acutely activating the ACC-LH pathway. Additionally, previous studies have shown that disruption to normal ACC activity suppresses motivation to obtain a high-value reward *only* when there is another low-value reward freely available (Hart et al., 2017, 2019). The results of our two-bottle preference test are consistent with this idea, and suggest that the ACC-LH circuit may be particularly relevant when an animal is presented with food options of unequal hedonic value. Moreover, there is both anatomical and functional evidence to suggest the ACC activity represents the value of competing options (Hart et al., 2019; Shenhav et al., 2016).

Neuroimaging studies in humans have implicated altered ACC activity in conditions of both overweight, such as obesity and obesity with food addiction, and underweight, including anorexia and bulimia (Brooks et al., 2013; De Ridder et al., 2016; Ellison et al., 1998a; Harding et al., 2018; Uher et al., 2004). When healthy individuals are asked to choose between healthy and unhealthy, high calorie foods there is increased

activation of the ACC (Harding et al., 2018), supporting the idea that the ACC encodes value differences of food options. In obesity, individuals may be more sensitive to the rewarding properties of food and may value unhealthy food higher than healthy food as a result, driving behaviour towards overconsumption of energy dense foods. While, in anorexia the anxiety associated with high calorie foods, may perturb the valuation of food options based on naturally rewarding taste properties and calorie content, and drive behaviour towards the intake of lower calorie foods. Intriguingly, a large number of animal studies implicate the ACC in the perception and neural representation of physical and psychological pain (Bliss et al., 2016). This highlights that the ACC is an important site integrating somatosensory, visceral and emotional information – all of which are incorporated when individuals are making food choices. Our results indicate that the ACC to LH circuit is likely to be a key pathway involved in the top-down regulation of food intake as ACC inputs to the LH suppress feeding. Future studies will focus on further delineating the role of this circuit in food choice and motivated food seeking behaviours.

5. Chapter Five: Overall discussion and conclusion

5.1 Summary

In studies presented here, we have investigated both homeostatic and non-homeostatic control of feeding and behaviour. In chapter one, we find that the hunger-sensing Agrp neurons promote adaptive behavioural and endocrine responses to acute stressors that would facilitate the survival of a hungry animal during the search for food in a dangerous and unfamiliar environment. The Agrp projections to regions such the LH, PVN and amygdala are likely to be key sites integrating homeostatic information in order to alter the behavioural and hormonal response to stress, although we did not directly test the influence of individual Agrp circuits here. Chapters two and three identified two novel anorectic cortical-hypothalamic circuits, in which ablation of these circuits increases consumption of a palatable diet and promotes body weight gain; while acute activation of these circuits suppresses feeding and motivated food seeking. Both circuits, projecting directly from regions within the prefrontal cortex to the LH, were able to elicit top-down control over homeostatic signals to restrict food intake.

5.2 Homeostatic control of food intake

Our work in chapter one highlights a role for Agrp neurons in mediating an adaptive response to an acute stressor. Our findings are consistent with a number of key studies that have investigated the behavioural effects of Agrp neuronal activation (Alhadeff et al., 2018; Burnett et al., 2016; Dietrich et al., 2012, 2015; Jikomes et al., 2016; Padilla et al., 2016). Additionally, our work builds on the current understanding of the role of Agrp neurons and demonstrates that Agrp activity prior to an acute stressor suppresses anxiety-like behaviour, improves memory recall and potentiates the HPA-axis response to stress. We also show that Agrp neurons promote adaptive behavioural responses to stress in a manner that is not dependent on simultaneous increases in plasma CORT. Thus, we conclude that the hunger-sensing Agrp neurons do not simply promote food intake, but rather a set of behaviours and physiological processes that together facilitate food seeking and lead an animal to food, even when under potential threat in stressful situations.

5.3 Non-homeostatic control of food intake

Historically, the LH has been identified as a key site involved in both homeostatic and non-homeostatic, or hedonic control of food intake (Hoebel & Teitelbaum, 1962; Teitelbaum & Epstein, 1962). Intra-hypothalamic inputs to the LH from the ARC, PVN and VMH encode information relevant to metabolic state. However, the LH also receives inputs from brain regions involved in reward processing, such as the ventral striatum, ventral pallidum and BNST that influence motivated food seeking and hedonic processes (Castro et al., 2015). While the intra-hypothalamic and mesolimbic inputs to the LH are well described, less is known about the direct inputs to the LH from the cortex and how these may ultimately control homeostatic and non-homeostatic drives to eat. In chapters two and three, we describe the function of direct mPFC and ACC to LH circuits in feeding and reward seeking behaviours.

Our results clearly show an anorectic role for the mPFC-LH and ACC-LH circuits, despite the well-described roles of the PFC and ACC in reward-seeking, cue-potentiated feeding and value-based decision making. Ablation of either circuit increases the consumption of a palatable diet and increases bodyweight gain. While, activation of either circuit suppresses food intake following a short-term fast, demonstrating that both circuits elicit top-down control over the homeostatic feeding drive. In addition to this, activation of the mPFC-LH circuit suppresses operant responding for sucrose pellets and is aversive. Our results are strikingly similar to the recent study from Wu *et al* (2020) who demonstrate that the right anterior insular cortex inputs to the LH reduce both appetitive and consummatory behaviours in fasted mice, and encode aversion (Wu et al., 2020). In this study, the right anterior insular was activated in response to aversive visceral stimuli suggesting that this circuit may suppress feeding under emergency conditions (Wu et al., 2020). Collectively, our results and those of Wu *et al* (2020) suggest that a novel direct integrated network of cortical inputs to LH circuits elicit top-down control to override homeostatic signals to eat and suppress food intake (Figure 5.1).

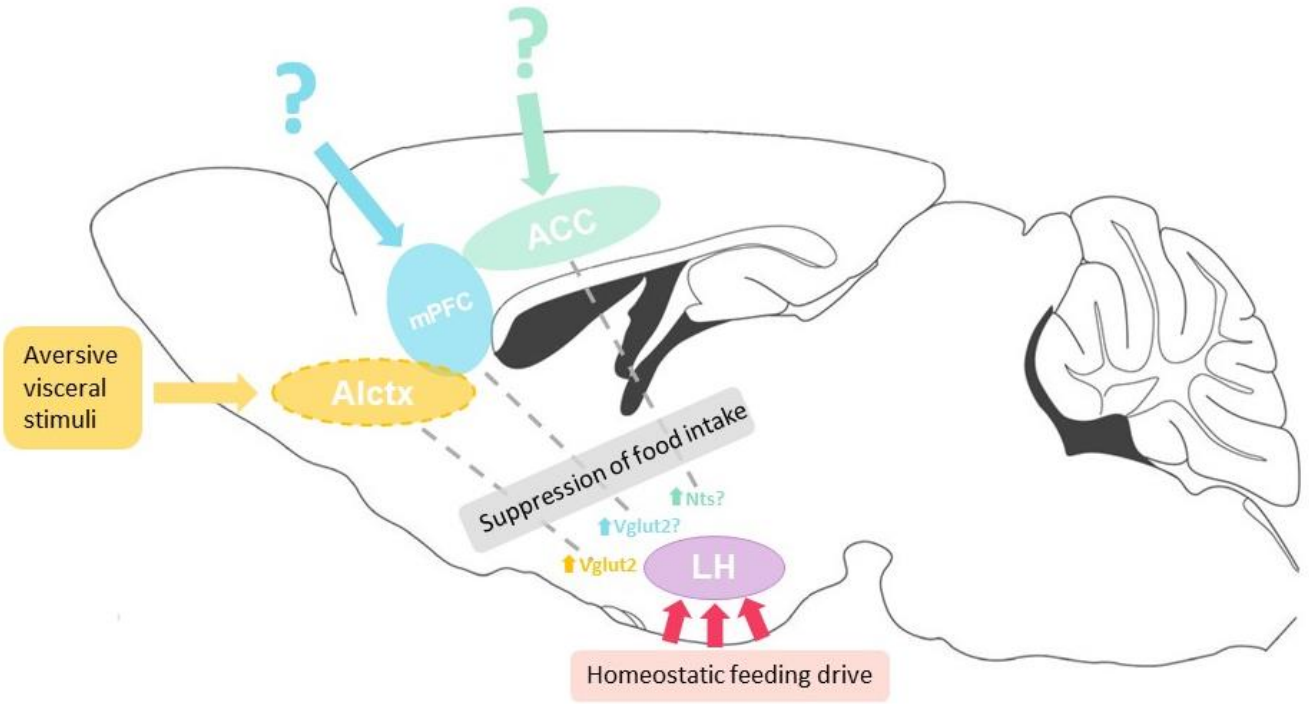


Figure 5.1 Cortical inputs to the lateral hypothalamus override homeostatic signals and suppress feeding. Inputs from the anterior insular cortex, as described in Wu *et al* (2020), and inputs from the medial pre-frontal cortex and anterior cingulate cortex that we describe here act to suppress food intake despite conflicting homeostatic signals. Wu *et al* (2020) report that the anterior insular cortex is active in response to aversive visceral stimuli, however the sensory or environmental information that would activate the medial pre-frontal cortex and anterior cingulate cortex inputs into the lateral hypothalamus remain unknown. The anterior insular cortex projections are excitatory and stimulate Vglut2 expressing neurons in the LH (Wu *et al* 2020). While we have not confirmed the downstream target neurons of the medial pre-frontal cortex or anterior cingulate cortex LH projecting neurons, we hypothesis that they may target predominantly Vglut2 and Nts expressing neurons respectively. Abbreviations: Anterior cingulate cortex (ACC); Anterior insular cortex (AIctx); Lateral hypothalamus (LH); Medial pre-frontal cortex (mPFC); Neurotensin (Nts); Vesicular glutamate transporter 2 (Vglut2).

However, there were some differences observed between the mPFC-LH and ACC-LH circuits. Firstly, in the chronic ablation studies the mPFC-LH circuit ablation resulted in body weight gain on a chow diet, without a detectable change in chow intake, suggesting that this circuit might influence LH-mediated control of energy expenditure, which has been described in the literature (Pei et al., 2019). In contrast, the ACC-LH circuit

ablation increased chow intake over a 4-day feeding period but did not result in significant body-weight gain over the 14-week chow-feeding period. While ablation of either circuit increased palatable food intake, ACC-LH circuit ablation reduced cumulative intake of sweet-solutions when mice had *ad lib* access to chow – indicating this circuit may be differentially sensitive to the hedonic properties of sweet-taste, versus sweet-taste and fat content. Finally, acute ACC-LH circuit activation significantly increased locomotor activity in all behaviour arenas tested. One explanation for these differences could be the LH neuronal population that each cortical circuit targets. We postulate that the mPFC to LH projection neurons primarily target the LH^{Vglut2} expressing neurons, as manipulation of the mPFC-LH circuit mirrors the phenotype observed with the manipulations of LH^{Vglut2} neurons (Jennings et al., 2013; Stamatakis et al., 2016). This idea is consistent with the findings of Wu *et al* (2020) who demonstrate that the anterior insular projects to LH^{Vglut2} neurons (Wu et al., 2020) and with previous studies showing that manipulation of LH^{Vglut2} neurons does not affect locomotor activity (Stamatakis et al., 2016). Given that ACC-LH circuit activation results in an increase in locomotor activity, it is unlikely that the ACC to LH neurons target only the LH^{Vglut2} neurons. Studies chemogenetically activating the LH population of *Nts* expressing neurons report a suppression of chow intake in fasted animals and an increase in locomotor activity in behavioural arenas (Woodworth, Beekly, et al., 2017). These results fit well with our observations of chemogenetic ACC-LH circuit activation and suggests that the ACC-LH projection neurons may target LH^{Nts} neurons, either exclusively or as well as LH^{Vglut2} neurons. Activation of LH^{Nts} using either chemogenetics or optogenetics does not result in aversive or appetitive behaviours (Woodworth, Beekly, et al., 2017). Therefore, a key experiment that will give more insight as to the downstream targets of the ACC-LH projecting neurons will be using optogenetics to activate the ACC-LH circuit during a real-time place preference task.

Our results are supported by human neuroimaging studies, which report that the mPFC, ACC and insula are key regions involved in the response to food cues, in both conditions of obesity and anorexia (Ellison et al., 1998a; Harding et al., 2018; Huerta et al., 2014; Stoeckel et al., 2008; Uher et al., 2004). It will be important to consider in future studies if these three regions communicate with each other to control feeding behaviour via the LH. This would suggest some functional redundancy within cortical projections to the LH, and could explain the reduced impact of chronic mPFC-LH and ACC-LH circuit ablation that we see here. Acute activation of each circuit would not allow for functional redundancies or re-organistation of cortical to hypothalamic circuits to occur.

Moreover, our findings support the notion that the LH is a key site of homeostatic and environmental information and an important region in the control of feeding and reward seeking. The LH receives input from a broad range of inputs from cortical, striatal, thalamic and hypothalamic regions (Berthoud & Münzberg, 2011; Clarke et al., 2018; Gabbott et al., 2005; Stuber & Wise, 2016). The inputs from Agrp neurons of the ARC to the LH represent a direct line for homeostatic information to rapidly increase feeding and influence motivated behaviour when required. At the same time, it is clear that cortical inputs to the LH can act to suppress homeostatic signals and modify both appetitive and consummatory behaviours. Potentially, in conditions of anorexia and obesity the cortical inputs to the LH are disordered allowing homeostatic signals

to be ignored, and environmental or hedonic information to take priority. For example, homeostatic signals that should increase intake, such as the appetite stimulating hormone ghrelin and Agrp, are significant elevated in anorexic patients (Moriya et al., 2006; Tolle et al., 2003). Despite these elevated homeostatic signals, anorexic patients still tightly control feeding and reward-seeking behaviour. In contrast, obese patients report a lack of control over consumption of palatable foods regardless of ample stores of body fat, and reduced sensations of hunger (Herbert & Pollatos, 2014). Potentially, genetic and/or environmental factors contribute to the normal functioning of the LH circuits that control feeding and reward-seeking behaviours.

5.4 Future directions

One limitation of the work presented herein is that we have not yet investigated how the cortical inputs into the LH impact on homeostatic feeding circuits within the hypothalamus. Future experiments, where we record from Agrp and POMC neuronal occurs during activation or inhibition of cortical to LH circuits, will be important to determine if these circuits influence the canonical hunger and satiety neurons. Additionally, our experiments so far have not addressed environmental or interoceptive cues that might lead to the activation of the mPFC-LH or ACC-LH circuits. To investigate endogenous neural activity of these circuits, retrograde delivery of the fluorescent calcium indicator GCaMP from the LH to mPFC and ACC using fibre photometry and miniscopes will be important. Wu *et al* (2020) report that the insular cortex is active in response to aversive visceral stimuli, which suggests the mPFC, and ACC to LH projection neurons could also be activated by aversive or stressful internal or environmental cues (Wu et al., 2020). These cortical regions are likely responsive to immediate threats or danger and may shut down food-seeking or foraging behaviours in order to promote survival, unless a homeostatic signal is already active, or is active for a duration long enough to counteract this – which is the model we present in chapter one.

In chapters two and three, we investigated two brain regions that appear to be differentially activated in conditions of both underweight and overweight. Our results suggest that targeting either the mPFC-LH or ACC-LH circuits in obesity or anorexia may yield therapeutic results – although the technology required to achieve pathway specificity may not be available for some time. Intriguingly, some studies exist to support this idea. In a patient with chronic anorexia, bilateral inhibition of the ACC using stereotactic radiofrequency thermocoagulation was successful in improving anorexia symptoms, without affecting cognitive capacity at both 5 years and 10 years post-surgery (Guerrero Alzola et al., 2019). In another study, transcranial magnetic stimulation of the dorsal ACC was reported to reduce alcohol cravings in a patient with severe alcohol dependence (De Ridder et al., 2011). While these studies are promising, much larger patient numbers are required to investigate both the efficacy and safety of these potential therapies. However, broad-spectrum activation or inhibition of the mPFC or ACC in humans may not be efficacious due to the opposing action of subpopulations of neurons in each region (Hart et al., 2019; Otis et al., 2017b). Our results highlight the need for further detailed animal studies that can specifically map the appropriate neuronal circuits, as well as the subpopulations of neurons within these circuits that may contribute to feeding related disorders. Future

technology that enables pathway specific manipulations in humans must be developed to minimise undesired effects of global stimulation or inhibition of brain regions involved in obesity or anorexia.

5.5 Conclusion

The three studies presented here add to the current understanding of the neural control of food intake and behaviour. We have demonstrated the behavioural effects of stimulation of bottom-up feeding circuits that use homeostatic information to guide appropriate behavioural and physiological responses to stress. In addition, we report the presence of two new top-down circuits that regulate feeding and behaviour. Potentially, these cortical to hypothalamic circuits are integrated and act to guide behaviour away from homeostatic needs and towards appropriate behaviours required to respond to urgent environmental or internal signals. Future work aims to: 1) determine the stimuli that trigger mPFC and ACC to LH circuit activation; 2) determine how our knowledge of the cortical to hypothalamic circuits can be utilised therapeutically to treat disordered responses to homeostatic signals that exist in both conditions of overweight and underweight; and 3) bridge the gap between the experimental examination of neural circuits controlling behaviour and human neuroimaging studies.

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