Histaminergic regulation of appetite

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Abstract

Histaminergic regulation of appetite

Food intake is essential to all animals. However, when energy consumption through food overtakes energy expenditure, obesity can result. Obesity has been identified as a worldwide health problem associated with diseases such as type 2 diabetes and hypertension. Thus, it is essential to find effective anti-obesity therapies. The aim of this thesis was to determine whether the histaminergic system could be pharmacologically manipulated to alter food intake and whether in particular the H_3R is a suitable therapeutic target. Histamine is a central neurotransmitter that plays a major role in controlling energy balance by acting through specific hypothalamic sites. Injections of histamine receptor-1 (H_1R) antagonists into the ventromedial hypothalamic nucleus (VMN) cause hyperphagia, whereas antagonism of presynaptic histamine receptor-3 (H_3R) causes hypophagia, leading to the hypothesis that selective antagonists or inverse agonists might be potential treatments for obesity through their actions on central H_3R. My aim was to assess the precise mode of action of histamine and H_3R drugs to affect acute, appetitive behaviour. Using feeding and behavioural studies I demonstrated the acute anorexigenic actions of histamine (ICV) and the H_3R inverse agonist, thioperamide (ICV or IP), in rats without disrupting the behavioural satiety sequence. In accordance with predictions, the H_3R agonist, imetit (ICV or IP), increased feeding. The actions of both thioperamide and imetit were blocked by the drug proxyfan, which in our model is acting as a neutral H_3R antagonist. Interestingly, both thioperamide and imetit caused anorexia in mice. C-Fos functional immunostaining revealed that systemic administration of thioperamide and imetit increased the activity of neurones in the key feeding nuclei of the hypothalamus, including the VMN. To further investigate the mode of action of histaminergic drugs, I carried out extracellular electrophysiological recordings from neurones of the rat VMN *in vitro*. Of the 197 VMN cells recorded, 62% were histamine-responsive, with 97% of these showing an increase in neuronal firing rates in response to histamine. The excitatory response to histamine was blocked in 90% of instances by pyrilamine, a selective H_1R antagonist. Neurones that responded to histamine previously were treated also with thioperamide. 88% of these neurones also responded with an increase in firing. The effect of thioperamide was blocked in all cases by co-administrating pyrilamine, proving that H_3R in the VMN are presynaptic autoreceptors, rather than heteroreceptors modulating the release of other transmitters. Imetit had an inhibitory effect on VMN neuronal firing in 86% of recorded cells. Proxyfan was able to block the changes in neuronal firing that both thioperamide and imetit caused. This suggests it is acting as a neutral H_3R antagonist in both our *in vivo* and *in vitro* models. Thus, using a neutral H_3R antagonist we have proven the effects imetit and thioperamide had on feeding and neuronal VMN firing were a direct result of activating a H_3R and, therefore, these compounds are receptor-specific for the H_3R. In conclusion, our results support a role for histaminergic receptors, including postsynaptic H_1R and presynaptic H_3R autoreceptors in the VMN, to modulate feeding.

Declaration

I, the undersigned declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Rachel Heather Clapp

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Chapter 1:

General Introduction

General Introduction

Food is essential to all animals and physiological states associated with energy balance (e.g., hunger and satiety) are major determinants of eating behaviour (Beaver *et al.,* 2006). By ingesting food, an animal gains energy to survive and maintain its internal environment. However, food intake must be balanced with energy expenditure otherwise over eating coupled with a lack of exercise can result in obesity. Obesity has been identified as a worldwide public health and economic problem affecting all age groups. It is associated with diseases such as type 2 diabetes, hypertension and hyperlipidemia (Masaki *et al.,* 2006). Genetic and environmental factors influence the development of obesity, yet the molecular mechanisms that are involved in obesity have not yet been determined (Blissmer *et al.,* 2006). As a consequence of increasing cases of obesity worldwide, numerous investigations have been carried out to determine the multiple factors at the origin of this imbalance between energy intake and energy expenditure (Beck, 2000). Attention was first focused on the nutritional aspects such as the use and storage of ingested food but, more recently, research has focused on the central nervous system (CNS) and its involvement in energy homeostasis.

Over the last few decades we have witnessed a huge advance in the study and the understanding of the central mechanisms and pathways involved in regulating food intake and the control of energy balance. One of the key discoveries over the years has been and the acceptance of the prominent involvement of the hypothalamus in the control of feeding behaviours. The hypothalamus plays an important physiological role in regulating energy balance and has a central role in the development of obesity (Morimoto *et al.,* 2001).

The overall aims of this project were to determine the effects of histamine, a neurotransmitter within the CNS associated with the regulation of appetite, on feeding and to describe the central mechanisms which might be targeted by potential therapeutic agents.

In this chapter I will first give an overview of the central and peripheral mechanisms controlling appetite and energy expenditure and then go on to discuss the histaminergic system and the role it plays in the regulation of feeding and the possibility of manipulating this system for the pharmaceutical control of appetite and body weight.

1.1 Central regulation of appetite

The control of food intake and energy metabolism is an extremely complicated process which depends on the brain's ability to receive, interpret and integrate a wide range of signals which indicate to the organism its nutritional state and energy level, and to make appropriate adjustments in the intake of food, energy expended and metabolism (Williams *et al.,* 2000). Body weight is regulated by complex interrelationships between central and peripheral factors. The CNS controls satiety, hunger and hedonistic drive. The peripheral nervous system (PNS) controls the metabolism and energy utilization associated with peripheral tissue, but is regulated by the CNS. These central and peripheral components of weight control are mediated by a multitude of neurotransmitters and hormones and their respective receptors (Williams *et al.,* 2000). Peripheral signals are sent to central neurones in specific areas of the brain known to control food intake and body weight (Jobst *et al.,* 2006).

By whatever means central neurones receive information regarding energy status, it is clear that the effects of peripheral signals are mediated by specific transduction systems in identifiable areas of the brain known to control food

intake and body weight (Jobst *et al.,* 2006). The hypothalamus is one of these brain areas and has been identified as having an important role in controlling food intake and energy expenditure. From brain lesion studies, the ventromedial hypothalamic nucleus (VMN) and paraventricular nucleus (PVN) are regarded predominantly as satiety centres, and the lateral hypothalamus (LH) as a feeding centre (for review see King, 2006) (Figure 1.1). After the discovery of these centres, several new areas located mainly in the hypothalamus have also been associated with food intake. The most important include the hypothalamic arcuate (ARC), dorsomedial (DMN) and suprachiasmatic (SCN) nuclei. They form, along with the VMN, PVN and LH, complex networks for the regulation of energy intake and expenditure (Beck *et al.,* 2000).

Appetite or the sensation of hunger is the leading force in the drive to eat. However, it is also controlled in a circadian fashion by a biological clock located within the suprachiasmatic nucleus (SCN). The SCN consists of two small, round nuclei resting dorsally on the optic chiasm either side of the third ventricle, that act as the internal clock within the body (Nagai *et al.,* 1978). The SCN responds to daily and seasonal light cycles to anticipate changes in the local environment and ensure changes in behavior, such as the organisms' ability to coordinate daily patterns in activity, feeding, energy utilization and energy storage, to maintain the optimum chance of survival (Bechtold DA, 2008). The pattern of eating behaviour is a highly-regulated phenomenon in all living organisms (Kalra *et al.,* 1999) and lesion studies have shown damage to the SCN results in loss of regulated feeding (Nagai *et al.,* 1978).

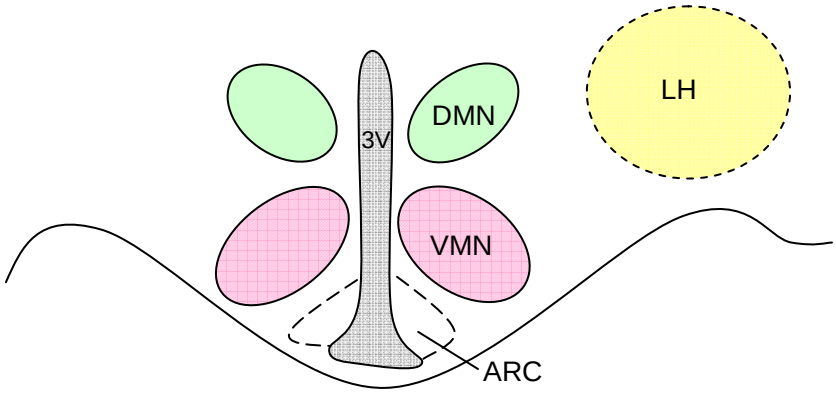

Figure 1.1.; A schematic diagram showing a coronal section through the hypothalamus at the level of the ventromedial nucleus (VMN). Other hypothalamic nuclei present at the level of this area are; dorsomedial nucleus (DMN), lateral hypothalamus (LH) and arcuate nucleus (ARC). 3V denotes the third ventricle.

1.2 The homeostatic pathways involved in regulating appetite

The regulation of food intake is very complex and organized by a complicated loop system involving humoral signals and afferent neuronal pathways to the brain, which are processed within the hypothalamic neuronal circuitry, and with commands then being sent back through vagal and spinal neurones to the body (Dozio *et al.,* 2007). Body weight is regulated by these complex interrelationships between central and peripheral factors (Ahima *et al.,* 2008).

Over the last few decades more and more experiments have investigated the role of the neuropeptides present in the areas associated with controlling appetite and feeding behaviours. These neuromodulators include peptides that inhibit or stimulate feeding behaviour. Corticotropin-releasing factor (CRF), cholecystokinin (CCK), neurotensin, cocaine- and amphetamine-regulated transcript (CART), α-melanocyte-stimulating hormone (α-MSH), pro-opiomelanocortin (POMC) and vasopressin are all anorexigenic and decrease feeding, whereas neuropeptide Y

(NPY), galanin, agouti-related protein (AgRP), melanin-concentrating hormone (MCH), and the orexins all stimulate food intake and are, thus, referred to as orexigenic (Beck *et al.,* 2000).

The ARC, situated at the base of the hypothalamus either side of the third ventricle, is important in the control of appetite. The ARC has been shown to contain the largest co-expression of the orexigenic peptides NPY and AgRP (Dhillo WS., 2007). Although research has also revealed a large population of cells within the ARC express anorectic signals, including CART and POMC (Elias *et al.,* 1998). The ARC neurones receive information regarding energy status from peripheral circulating factors, such as ghrelin from the stomach, passing the blood-brain barrier (Schwartz *et al.,* 2000). The orexigenic and anorexigenic neurones in the ARC project into a number of other hypothalamic sites involved in appetite, including the VMN, DMN, LH, and the PVN, allowing the signals from the peripheral system to be processed in the necessary brain areas and body homeostasis to be maintained (Harrold *et al.,* 2006) (figure 1.2).

The LH receives extensive connections from the ARC including both orexigenic and anorexic inputs from the NPY/AgRP and POMC/CART neurones (Dhillo WS., 2007). This hypothalamus is the site of production of the orexigenic neuropeptides, MCH and the orexins (Sakurai *et al.,* 1998).

Although electrolytic lesions in the DMN have been shown to disrupt feeding to a much lesser degree than those of the VMN (Kalra *et al.,* 1999), suggesting a less pivotal role in the control of food intake, microinjection of orexigenic compounds into the DMN elicits feeding (Li *et al.,* 1998). Yokosuka *et al.* illustrated that the inhibition of NPY-induced feeding by leptin administration, increased neuronal c-Fos in the DMN, which acts as a marker of neuronal activation (Yokosuka *et al.,* 1998). Prominent ARC efferents project to the DMN and a large number of DMN efferents project to both the VMN and PVN (Kalra *et al.,* 1999) illustrating the complex and multiple connections these areas have with

each other and showing the complex network systems involved in the control n feeding.

The PVN is situated in the dorsal region of the hypothalamus. It contains a dense and heterogeneous cluster of neurones that extend out either side of the third ventricle within a well-defined area (Sawchenko *et al.,* 1993). Research suggests these vast neuronal elements contained within the PVN are involved in the control of ingestive behaviour. Microinjection of virtually all the known orexigenic signals, including, NPY, GAL, orexins, GABA, opioids, noradrenaline, and adrenaline, into the PVN stimulates feeding (Xu *et al.,* 1995). Whereas microinjection of a number of anorexigenic neuropeptides, such as CRH and leptin, cause a significant reduction in food intake. Additionally, high densities of c-Fos-positive neurones have been shown within the PVN in response to administration of both orexigenic and anorexigenic peptides (Dhillo WS., 2007). These findings coincide with other evidence that suggests the PVN is one of the crucial sites for the release of both orexigenic and anorexgenic signals (Kalra *et al.,* 1999).

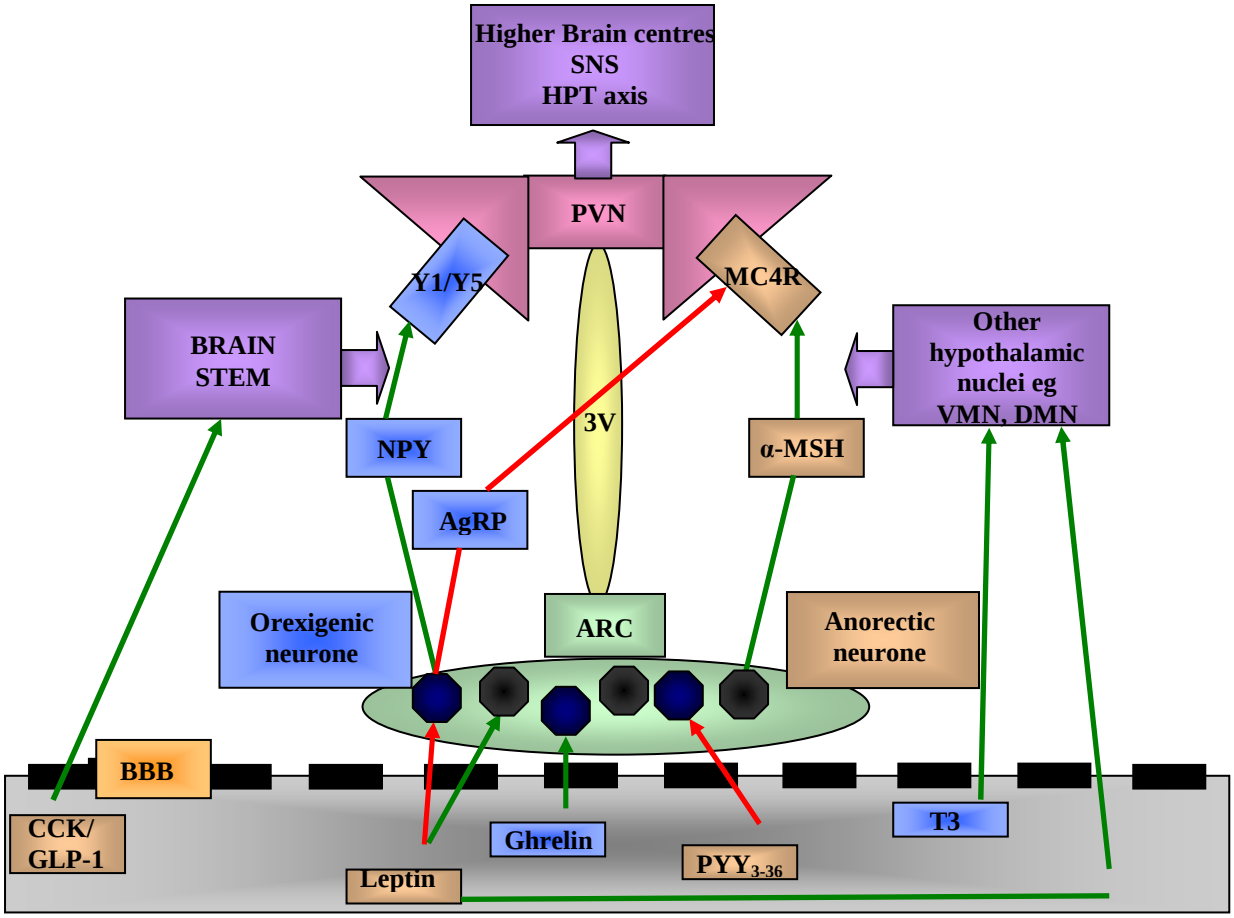

Figure 1.2.; Adapted from DhillO, 2007. Schematic diagram the circulating factors and central appetite circuits involved in the regulation of appetite. Peripheral circulating factors activate circuits within the hypothalamus and brainstem mediating their effects on food intake. There are extensive reciprocal connections between the hypothalamus and the brainstem with energy intake being coordinated based on the information received by both regions. PVN: paraventricular nucleus; 3V: third ventricle; ARC: arcuate nucleus; VMN: ventromedial nucleus; BBB: blood–brain barrier; SNS: sympathetic nervous system; HPT axis: hypothalamo-pituitary-thyroid axis; Y1-Y5: Y1-Y5 NPY receptors; MC4R: melanocortin 4 receptor; NPY: neuropeptide Y; AgRP: agouti-related protein; α-MSH: alpha melanocyte-stimulating hormone; CCK: cholecystokinin; GLP-1: glucagon-like peptide-1; PYY: peptide YY; T3: triiodothyronine. Appetite-regulating factors in blue stimulate and those in black inhibit food intake. Green arrows represent activation, and red arrows represent inhibition of the pathway.

1.3 VMN regulation of appetite

As described above, the hypothalamus has an important role in controlling food intake and energy expenditure. To further support this, evidence obtained from clinical presentations in hypothalamic tumour patients and from preclinical lesion studies suggest that damage to the mediobasal hypothalamic areas, in particular the VMN, are associated with increased food intake, morbid obesity and insulin resistance, while damage to more lateral hypothalamic structures are associated with anorexia and adipsia (Anand *et al.,* 1951). Neuroimaging studies have illustrated neurones within the immediate area of the VMN become activated during feeding (Liu *et al.,* 2001). Electrical stimulation of the VMN decreases feeding, whereas stimulation of the LH region increases appetite (Mittleman *et al.,* 1984). It has even been noted that rats with VMN lesions often begin eating insatiably, even before fully recovering from the effects of anesthesia (Brobeck *et al.,* 1943, Brooks *et al.,* 1946). Brooks *et al.* suggested the overeating and obesity resulting from VMN lesions can be crudely divided into two stages; firstly, a dynamic phase of marked hyperphagia and rapid weight gain, which is then followed by a reduction in food intake as body weight levels off and is then maintained during the static second phase of obesity (Brooks *et al.,* 1946). Thus, the VMN is a “satiety centre” of the brain and therefore, if damaged, it can no longer enforce feeding restraints, resulting in overeating and weight gain (Sclafani A., 1971, Bray *et al.,* 1981). The VMN has direct links with the PVN and DMN and via these it connects indirectly with the LH (Harrold *et al.,* 2004), illustrating the complex circuitry surrounding the VMN involved in regulating food intake within the hypothalamus.

VMN lesion-induced obesity has been found in a wide variety of species, which include mice (Mayer et al., 1955), rabbits (Romanouk A., 1962), ground squirrels

(Mrosovsky *et al.,* 1974), cats (Anand *et al.,* 1955), chickens (Lepkovsky *et al.,* 1966), sparrows (Chen *et al.,* 2006), dogs (Rozkowska *et al.,* 1971), monkeys (Brooks *et al.,* 1946) and humans (Bray *et al.,* 1979).

Research has illustrated that the VMN has a large number of glucose-sensing neurones that dynamically respond to hypo- or hyperglycaemia (Anand *et al.,* 1964, Oomura *et al*., 1973, Song *et al.,* 2001). Studies have also revealed that the VMN has many receptors that respond to neurotransmitters and hormones, such as dopamine (Davidowa *et al.,* 2002, Fetissov *et al.,* 2002, Meguid *et al.,* 1997), serotonin (Leibowitz SF., 1986, Meguid *et al.,* 2000), GABA (Dellouade *et al.,* 2001), insulin (Baskin *et al.,* 1999, Bruning *et al.,* 2000), leptin (Dhillo WS., 2007), histamine (Magrani *et al.,* 2004, Mollet *et al.,* 2003, Sakata *et al.,* 2003), ghrelin (Kamegai *et al.,* 2001), orexin (Heidel *et al.,* 1999), and estrogen (Pfaff *et al.,* 1973, Wade *et al.,* 1970) to affect feeding behaviour. The response of many of these receptors has been found to be abnormal in obese animals that were overfed since birth (King *et al.,* 2006). The VMN receives projections from ARC NPY-, AgRP- and POMC-immunoreactive neurones and in turn VMN neurones project to other hypothalamic nuclei, such as the DMN, and to brainstem regions, such as the nucleus of the solitary tract (NTS) (Wynne *et al.,* 2005). Mice with reduced brain-derived neurotrophic factor (BDNF) receptor expression or decreased BDNF signalling have been shown to significantly increase food intake resulting in increased body weight (Rios *et al.* 2001, Xu *et al.* 2003). Xu *et al*. demonstrated that BDNF is highly expressed within the VMN, and its expression is dramatically reduced by fasting (Xu *et al.* 2003) or by melanocortin agonists (figure 1.2). This suggests BDNF neurones within the VMN may form downstream pathways through which the melanocortin system regulates appetite and body weight (Wynne *et al.,* 2005). The VMN has also been identified as a key target for leptin, which acts on the hypothalamus to inhibit feeding and stimulate energy expenditure (Dhillon *et al.* 2006). Here leptin could be acting via cells containing

the VMN-expressed neuropeptide, pituitary adenylate cyclase-activating polypeptide (PACAP), which also reduce food intake and energy expenditure (Hawke *et al.,* 2009).

1.4 The brainstem and gut involvement in the regulation of appetite

The brainstem has numerous interactions with hypothalamic circuits and plays a major role in the regulation of energy homeostasis. In particular there are extensive reciprocal connections between the NTS and other brainstem areas (Ter Horst *et al.* 1986). The ARC in the hypothalamus is pivotal for receiving peripheral signals mediating information on appetite and metabolic needs, whilst the NTS is the key entry port for signals from the gastrointestinal (GI) tract (MacDougald *et al.,* 2002) (figure 1.3). The GI tract acts not only as a conduit for food, but is also crucial for the digestion and absorption of nutrients (Ahima *et al.,* 2008). The brain, and in particular the NTS receives signals from the GI tract about meal size and content through sensory nerves and via the circulation (Schwartz *et al.,* 2000 b). Projections from the NTS and the parabrachial nucleus in the brainstem innervate the PVN, DMN and ARC nuclei of the hypothalamus and the LH area, central nucleus of the amygdala (AMY) and bed nucleus of the stria terminalis (Ahima *et al.,* 2008). The NTS projects also to the visceral sensory thalamus, which goes on to communicate with the visceral sensory cortex, which mediates the conscious perception of fullness and satiety. Studies demonstrate a powerful negative feedback control of vagal afferent innervation on feeding (Schwartz *et al.,* 2000, Smith *et al.,* 1985, South *et al.,* 1988).

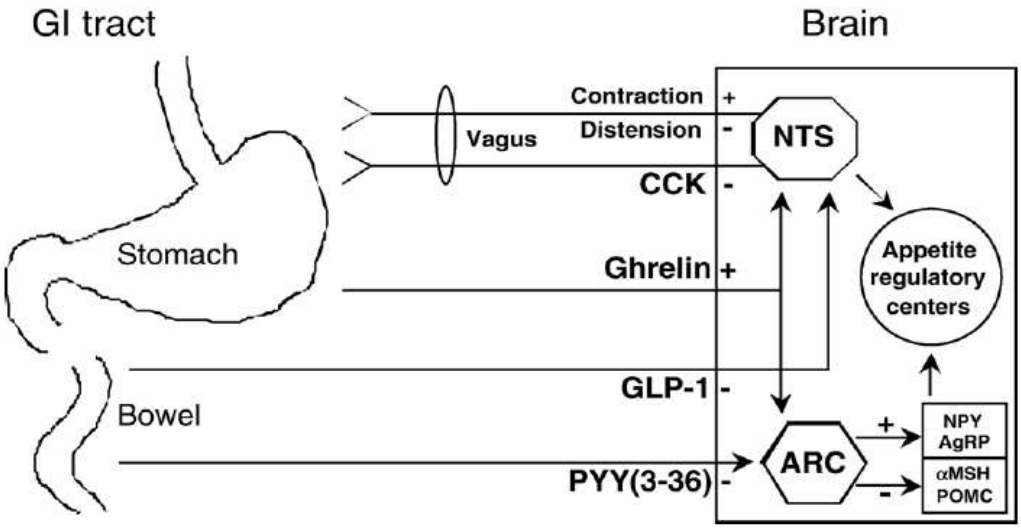

Figure 1.3.; Figure taken from Naslund *et al.*, 2007, Afferent gastrointestinal signals controlling food intake. CCK, cholecystokinin; GLP-1, glucagon-like peptide-1; PYY(3-36), peptide YY(3-36); NPY, neuropeptide Y; AgRP, agouti-related peptide; α-MSH, α melanocyte-stimulating hormone; POMC, pro-opiomelanocortin; NTS nucleus of the tractus solitarius; ARC, arcuate nucleus.

Many of the peptides involved in the regulation of energy homeostasis in the brain are also found in the enteric nervous system and enteroendocrine cells of the mucosa of the GI tract (Naslund *et al.*, 2007). Peptide signalling from the gut influences food intake, that then goes on to influence neuropeptidergic neurones in the brain. These hormones include CCK, which was the first gut secreted peptide to be identified as a satiety factor; GLP-1, a product of the preproglucagon gene secreted primarily by endocrine cells of the small and large intestine after eating: PYY, a gut peptide belonging to the same family as NPY and is released in response to feeding to cause a decrease in food intake; amylin,

which exerts anorectic effects; ghrelin, a growth hormone-releasing peptide that is predominantly released from the stomach and acts as a hunger signal: insulin which is secreted in response to meals and increases glycogen storage; and the endocannabinoids which have significant effects on appetite and metabolism along with glucocorticoids (Ahima *et al.,* 2008).

1.5 The reward pathways involved in regulating appetite

There are multiple interactions between the homeostatic and hedonic mechanisms controlling food intake (Zheng *et al.,* 2009). The rewarding nature of food can act as a stimulus to feeding, even when hunger and energy homeostasis are missing. Although the sensation of reward is ultimately influenced by energy status, the subjective palatability of food is altered in the fed state compared with the fasting state (Berridge *et al.,* 1991). Thus, signals of energy status influence reward pathways (Wynne *et al.,* 2005). The reward circuitry is complex and involves many interactions between numerous signalling systems (figure 1.4). Cognitive and emotional factors have an important role in regulating feeding, especially in humans (Zheng *et al.,* 2009). This high-level control involves integration of a number of external and internal signals, which include, peripheral signals related to energy status, food-related signals conveyed by taste, smell, and other senses, environmental cues, such as the sight of food, and memory of past feeding experiences (Benarroch *et al.,* 2010). Several functional neuroimaging studies indicate that the insular, orbitofrontal, and anterior cingulate cortical areas are activated in relationship to these stimuli and participate in motivational aspects of feeding behaviour in humans (Rolls ET., 2008, Zheng *et al.,* 2009). The nucleus accumbens is vital in the reward aspect of food intake as it integrates the homeostatic, hedonic, motivational, and cognitive aspects of food intake via its connections with the prefrontal cortex, amygdala, and lateral

hypothalamus (Kelley *et al.,* 2005). Although, it has also been recognised that the reinforcing effect is lost in the fasted state, suggesting homeostatic mechanisms can override hedonistic mechanisms (Hayward *et al.,* 2002, Wynne *et al.,* 2005).

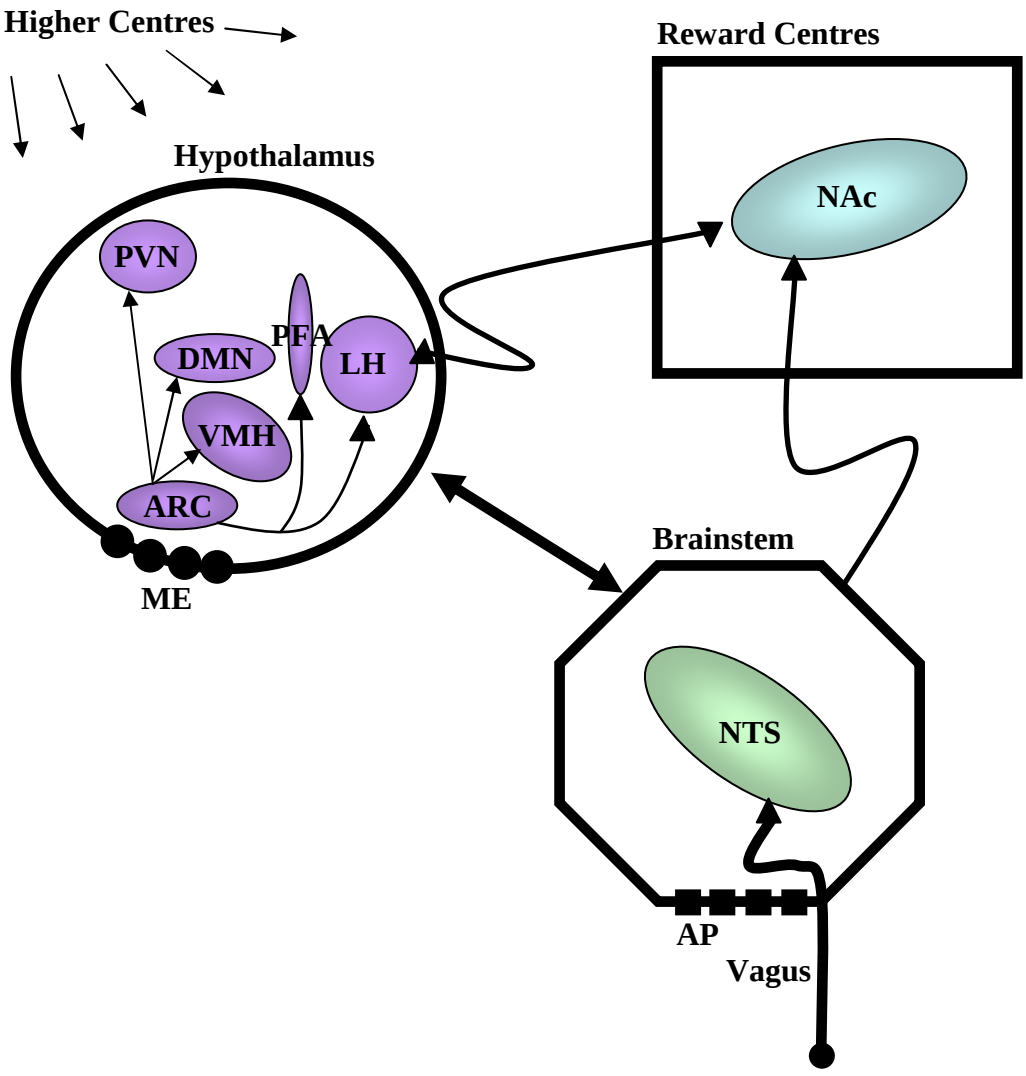

Figure 1.4.; Adapted from Wynne *et al.* 2005. This diagram illustrates the central control of appetite. AP, area postrema; ME, median eminence; NAc, nucleus accumbens; PFA, perifornical area.

Orexin neurones are important in linking energy balance, behavioural arousal, and reward. These neurones are active during wakefulness and participate in the short-term regulation of energy homeostasis (Sakurai T., 1999). Orexin neurones that activate ventral tegmental area dopaminergic neurones have been implicated in hedonic mechanisms of food intake (Harris *et al.,* 2006). Opioids also play an important role in feeding and reward behaviours. For example, Hayward *et al.* (2002) found that regardless of the palatability of the food tested, a lack of enkephalin or β-endorphin abolishes the reinforcing property of food in mice. The dopaminergic system is also integral to reward-induced feeding behaviour, for example, mice which lack dopamine have fatal hypophagia. Injections of opioid and dopamine agonists into the nucleus accumbens preferentially stimulate the ingestion of highly-palatable foods (Zhang *et al.,* 2003). Stratford *et al.* have suggested that GABA-ergic connections between the nucleus accumbens and the LH may mediate hedonistic feeding by disinhibition of LH neurones (Stratford *et al.,* 1999). Additionally, the MCH neurones in the LH may reciprocally influence the reward circuitry, as the nucleus accumbens expresses MCH receptors (Saito *et al.,* 2001). Other systems, including those mediated by endocannabinoids and serotonin, may also be able to modulate both reward circuitry and homeostatic mechanisms controlling feeding (Cota *et al.,* 2003).

Signaling systems expressed in the hypothalamus also contribute to both homeostatic and hedonic control of food intake are those containing CART peptide and the endocannabinoids (Harrold *et al.,* 2006). CART is colocalized with POMC in neurones of the ARC and exerts a potent anorexigenic effect in rodents (Dietrich *et al.,* 2009). Whereas the endocannabinoids promote feeding and provide anabolic signals via multiple actions at the hypothalamus, mesolimbic reward system and periphery (Bellocchio *et al.,* 2008). Additionally, leptin inhibits firing of dopaminergic neurones and feeding-induced dopamine release in the

nucleus accumbens (Hommel *et al.,* 2006), whereas ghrelin stimulates both dopamine release and feeding (Abizaid *et al.,* 2006).

1.6 The histaminergic system

While the role of histamine in neural functioning and behaviour is only partially understood, it is widely accepted that histamine functions as a central neurotransmitter (Orthen-Gambil *et al.,* 1992, Sakata *et al.,* 1997, Takahashi *et al.,* 2002, Zawilska *et al.,* 1985). In the mammalian CNS, anatomical, neurochemical, pharmacological and electrophysiological data have converged to demonstrate that histamine is involved in a wide array of neuroendocrine, cardiovascular and thermoregulatory functions, including arousal, cognition, locomotor activity, autonomic and vestibular functions, feeding and drinking, sexual behaviour and analgesia (Hough LB., 1988; Schwartz *et al.,* 1991; Wada *et al.,* 1991, Chotard *et al.,* 2002, Gomez-Ramirez *et al.,* 2002, Hancock *et al.,* 2004).

In the mammalian brain, histamine is synthesized by a population of neurones whose cell bodies are restricted to the tuberomammillary nuclei (TM) of the posterior hypothalamus (Schwartz *et al.,* 1991). Diffuse histaminergic nerve fibres consisting of long, varicose, arborizing, slowly-conducting axons project from the TM to virtually all parts of the brain (figure 1.5). These axons form synaptic contacts with other neurones, while varicosities can be apposed to neurones, glial cells and capillaries (Bugajski, *et al.,* 2003). The highest density of histamine fibres in the brain is in the ventral half of the posterior hypothalamus, including the VMN. Although the median eminence, SCN and PVN are also highly innervated with histaminergic fibres, all areas of the hypothalamus receive a moderate to strong histaminergic innervation (Gomez-Ramirez *et al.,* 2002).

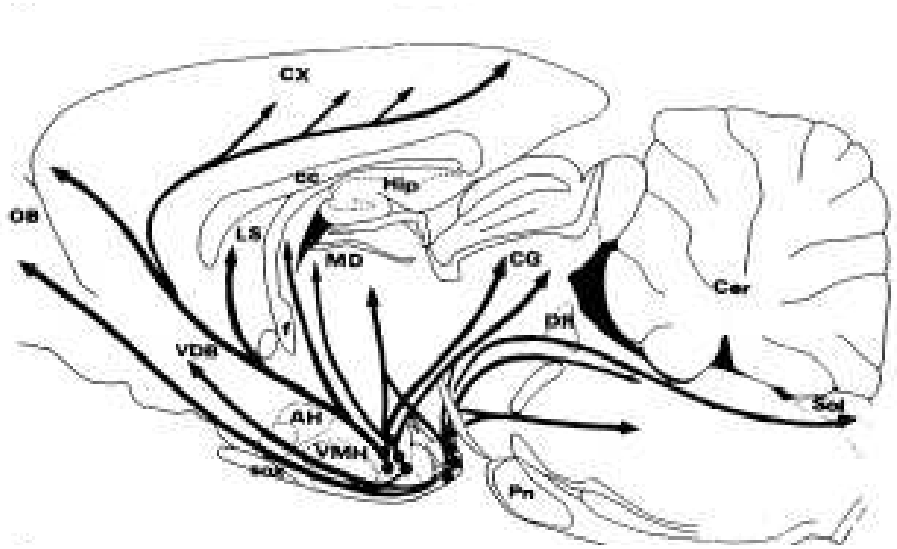

Figure 1.5.; Diagram taken from Schwartz *et al.,* 1990. The disposition of the main histaminergic pathways in the rat brain as indicated by the black arrows. The diagram illustrates the sagital section of the rat brain. AH, anterior hypothalamic area; cc, corpus callosum; Cer, cerebellum; CG, central grey; CX, cerebral cortex; DR, dorsal raphe nucleus; Hip, hippocampus; LS, lateral septum; MD, mediodorsal thalamus; MMN, medial mammillary nucleus; OB, olfactory bulb; Pn, pontine nuclei; Sol, nucleus of the tractus solitarius; Sox, supraoptic decussation; VDB, nucleus of vertical limb of diagonal band; VMN, ventromedial hypothalamic nucleus.

Cortically projecting histaminergic neurones share, along with other aminergic neurones, certain electrophysiological properties evidenced by extracellular recording. Histaminergic neurons fire spontaneously, slowly and regularly, and their action potentials are of long duration (Haas *et al.,* 1989). The rate of firing varies depending on the behavioural state of the animal. For example, direct recordings of histamine neurones in cats indicated their activity is high during waking and attention, but low or absent during sleep (Haas *et al.,* 2003). Thus,

histaminergic neurones exhibit a circadian rhythm in their firing rate (Schwartz *et al.,* 1991). Furthermore, in the rat hypothalamus, histamine levels are low, whereas synthesis is high during the dark period, suggesting neuronal activity is enhanced during the active phase (Hill *et al.,* 1997). In addition, in the mouse cerebral cortex, striatum and hypothalamus, tele-methylhistamine levels, a histamine analogue, are doubled at the end of the dark phase compared with the beginning of the light phase (Morisset *et al.,* 2000). Mochizuki *et al.* have also demonstrated that histamine release from the anterior hypothalamus of freely moving rats, evaluated by *in vivo* microdialysis, gradually increases in the second half of the light period and is maintained at a maximal level during the active phase (Mochizuki *et al.,* 1992).

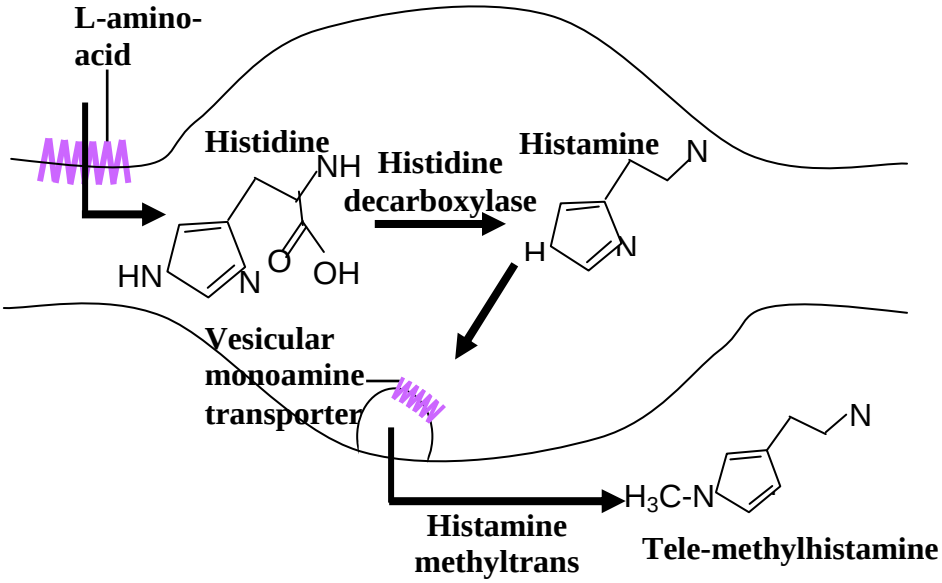

Figure 1.6.; This diagram has been adapted from Haas *et al.,* and illustrates histamine transport and metabolism in neurones. Histidine is brought into neurones via the L-amino-acid-transporter. Here histamine is synthesized by the specific enzyme histidine decarboxylase. Histamine is then taken up into vesicles by the vesicular monoamine-transporter VMAT-2. After release, histamine is methylated by histamine-methyltransferase, which is located post-synaptically in glia, to tele-methylhistamine. Tele-methylhistamine exhibits no histamine-like activity. The turnover rate for neuronal histamine is very high, and its half-life, which is normally about 30 minutes, can change quickly depending on neuronal activity. Increased neuronal activity, such as stressful situations increases histamine turnover (Haas *et al.,* 2003).

A vast body of evidence illustrates the importance of central histamine signaling in the control of food intake and energy regulation (Yoshimatsu *et al.,* 1993, Yoshimatsu *et al.,* 2001, Yoshimatsu H., 2006, Yoshimoto *et al.,* 2006, Gotoh *et al.,* 2007). Behavioural studies have revealed histamine suppresses food intake when administered centrally or systemically (Doi *et al.,* 1994, Endou *et al.,* 2000, Lecklin *et al.,* 1998, Lecklin *et al.,* 1995). Orthen-Gambill *et al.* suggested that removal of histamine's inhibitory effect might in turn stimulate

feeding (Orthen-Gambill *et al.,* 1992). They found in rodents, if histamine levels were decreased using the histamine synthesis inhibitor α-fluoromethylhistidine (α-FMH), which is a potent, highly specific, and irreversible inhibitor of histidine decarboxylase (HDC), an increase in both food intake and body weight was produced. Additionally, central histaminergic activity is increased by food intake after fasting (Itoh *et al.,* 1991). Yoshizawa *et al.* showed there were alterations within central histaminergic activity in anorexia nervosa patients (Yoshizawa *et al.,* 2009). Histamine depolarises select brainstem neurones in the NTS and dorsal motor nucleus of the vagus (Poole *et al.,* 2008), which together with the hypothalamus, have a fundamental role in the control and regulation of food intake (Jelsing *et al.,* 2009). In the GI tract, histamine is considered to regulate at least three major functions, firstly the enhancement of gastric acid production (Tari *et al.,* 1993), secondly, modulation of GI motility (Bolton *et al.,* 1981), and finally the alteration of mucosal ion secretion (Wang *et al.,* 1990). Thus, it seems endogenous histamine plays an important role in regulating food intake.

1.7 Histamine receptor types

Histamine exerts its multiple biological activities through the activation of at least four distinct receptors: H_1R, H_2R, H_3R and H_4R (Leurs *et al.,* 1995, Lovenberg *et al.,* 1999, Masaki *et al.,* 2006). All four histamine receptors belong to the superfamily of heptahelical seven-transmembrane (7TM), G-protein-coupled receptors (GPCR) (Govoni *et al.,* 2006). Constitutive activity has been shown in all the histamine receptors and is explained in more detail in Chapter 5 of this thesis.

Terao *et al.* suggest that histamine H_1R are located post-synaptically with high densities of these receptors being apparent in the hypothalamus and other

limbic regions (Terao *et al.,* 2004). H_2R are also mainly located post-synaptically and are found in the hippocampus, amygdala and basal ganglia. Histamine H_3R are located on the somata and axon terminals of histamine neurones where they serve as autoreceptors to modulate histamine synthesis and release, and are also located pre- and post-synaptically the brain. Histamine H_4R are expressed primarily in bone marrow and eosinophils, and are found at very low levels in the brain. However, as the H_4R are a more recent discovery considerably less research has been carried out on these receptors. The H_1R is a 486–491 amino acid protein encoded by an intronless gene (Yamashita *et al.,* 1991), and is coupled to the Gq/11 protein and phospholipase C. Like the H_1R, an intronless gene encodes the H_2R, and the protein consists of 358–359 amino acids. It is coupled to Gs and protein kinase A. Several isoforms of the H_3R, consisting of 326–445 amino acids, are derived from a single gene by alternative splicing (Lovenberg *et al.,* 1999). The H_3 autoreceptor is coupled to Gi/Go, displays significant constitutive activity, and controls histamine release and synthesis (Morisset *et al.,* 2000). The H_3R is highly heterogeneous and its gene structure is more complex than that of the H_1 and H_2 receptors. H_3R are coupled to Gi/o and high voltage-activated Ca^{2+} channels, a typical mechanism for the regulation of transmitter release (Haas et al., 2003. The H_3R is coupled negatively to cAMP and activates the mitogen activated protein kinase pathway (Drutel *et al.,* 2001). The human H_3R gene has four exons and gives rise to six or more alternative splice variants (Coge *et al.,* 2001). The H_3R regulates the release of several transmitters in brain areas such as the substantia nigra, amygdala and cerebral cortex (Haas *et al.,* 2003). The gene encoding the H_4R has a similar intron–exon arrangement as the gene encoding the H_3R (Coge *et al.,* 2001). The H_4R is a 390-residue GPCR encoded by three exons and is expressed predominantly in bone marrow, eosinophils and mast cells (De Esch *et al.,* 2005). H_1 and H_2 receptors have mostly excitatory actions on neurones or potentiate excitatory

inputs. Conversely, H_3R activation causes autoinhibition of TM neurones and inhibition of neurotransmitter release (Haas *et al.,* 2003). As H_4R are most similar to H_3R it could be assumed that they also exhibit inhibitory affects on their target cells. The H_1 and H_2 receptors are found densely throughout the central and peripheral nervous system, the H_3R is predominantly found within the CNS and the H_4R is found solely within the PNS. Sander *et al.* found that H_1R, H_2R, and H_4R are expressed in the human GI but found no evidence of H_3R expression here (Sander *et al.,* 2006).

1.7.1 The H_1 receptor

H_1R are located post-synaptically and are found in the hypothalamus, cerebral cortex, basal forebrain and limbic system (Morimoto *et al.,* 2001) (see figure 1.7). H_1R mediate excitatory actions on whole-brain activity. The classic antihistamines and antipsychotics act as H_1R antagonists but H_1R-based drugs have also been effective in treating depression, anxiety and sleep disorders. Stimulation of H_1R regulates the level of H_1R protein expression through activation of H_1R mRNA synthesis (Hill *et al.,* 1997).

Histamine H_1R knockout (H_1R-KO) mice (Inoue *et al.,* 1996) exhibit a complex cognitive phenotype, including both impairments and improvements in a variety of learning and memory tasks (Dai *et al.,* 2007, Zlomuzica *et al.,* 2008). The results of physiological and pharmacological studies have revealed that brain histamine and H_1R are involved in the regulation of feeding and obesity in rodents (Masaki *et al.,* 2006). The effects of the H_1R on weight gain were first witnessed in patients taking antipsychotic and antidepressant drugs with H_1R antagonistic properties which caused drastic and rapid weight gain. Research suggested that weight gain caused from taking antipsychotic drugs was through a drug-induced decrease in the hypothalamic expression of the H_1R, blockade of which is linked

to downstream AMPK activation, resulting in increased food intake which, when coupled with insufficient locomotion, results in drastic weight gain (Kim *et al.,* 2007, Han *et al.,* 2008).

Central administration of histamine can reduce body mass and adiposity in diet-induced and genetically-obese mice by altering food intake and energy expenditure. Endogenous histamine in the brain exerts an inhibitory effect on feeding behaviour through the H_1R (Fukagawa *et al.,* 1989; Sakata *et al*., 1994, Ookuma *et al.,* 1993, Doi *et al.,* 1994). However, H_1R antagonists can stimulate other neurotransmitter systems which affect feeding behaviour, such as serotonin or noradrenaline (Lidbrink *et al.,* 1971). Therefore, potential effects of H_1R antagonists on other transmitter systems should always be considered. Through site injections of histamine, H_1R in the VMN and PVN are implicated in the neuronal regulation of appetite (Masaki *et al.,* 2003). A simple, yet clear indication that endogenous H_1R are important in feeding and body weight regulation is provided by the fact that the H_1R-KO mouse is obese (Inoue *et al*., 1996). Although it must be noted that H_1R are also found in peripheral tissues that are concerned with regulating body composition (Masaki *et al.* 2006). Neuronal histamine and the H_1R are also involved in the central regulation of energy homeostasis through sympathetic influences on uncoupling protein expression in brown adipose tissue (Masaki *et al.,* 2001, Takasashi *et al.,* 2002). Consistent with these observations, Masaki *et al.* showed that H_1R-KO mice displayed late-onset obesity and decreased energy expenditure (Masaki *et al.,* 2004). Interestingly, the anorectic activity of ICV leptin was significantly attenuated in H_1R-KO mice (Masaki *et al.,* 2001), suggesting the obese phenotype of H_1R-KO mice could be explained in part by reduced sensitivity to leptin.

H_1R antagonists are not used pharmacologically for appetite regulation as they cause a number of adverse effects due to ubiquitous expression in peripheral tissues, including in lymphocytes, heart, and spleen, as well as in the CNS

(Kinnunen *et al.,* 1998). The H_1R is involved in a number of actions as it is so widely found peripherally and centrally and, therefore, it is difficult to make drugs that target just one of its many functions and pathways.

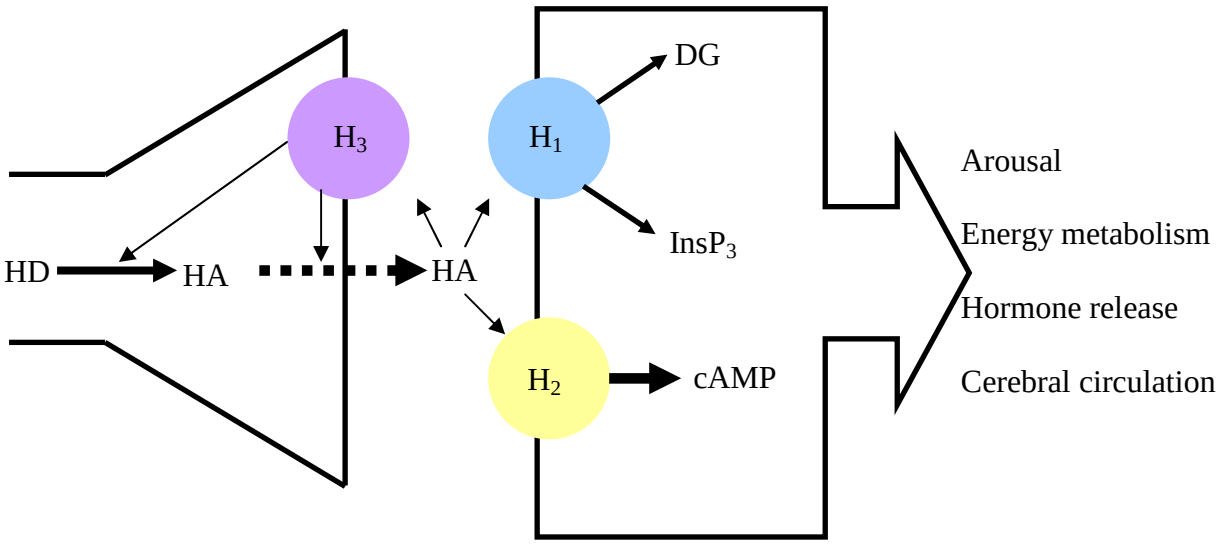

Figure 1.7.; Illustration based on Hill (1990) showing the interaction between histamine (HA) and presynaptic H_3 receptors on histaminergic nerve terminals, and H_1 and H_2 receptors on postsynaptic cells in the central nervous system. H_3-receptor stimulation leads to an inhibition of both the synthesis of histamine from histidine (HD) and its release into the synaptic cleft. H_1- and H_2-receptor stimulation leads to physiological responses via the action of intracellular messengers diacylglycerol (DG), inositol trisphosphate ($InsP_3$) and cyclic 3`,5` adenosine monophosphate (cAMP).

1.7.2 The H_2 receptor

H_2R like H_1R are also located post-synaptically (figure 1.7) and occur at high densities in the basal ganglia and parts of the limbic system, such as the hippocampal formation and amygdala (Masaki *et al.,* 2006). As there are diffuse projections of histamine fibres throughout the mammalian brain. Histamine is involved in the central control of a large number of behaviours and functions,

including neuroendocrine responses (Schwartz *et al.,* 1991) and cardiovascular functions (Onodera *et al.,* 1994), motion sickness, sleep and wakefulness cycle (Schwartz *et al.,* 1991), stress-induced responses and behaviour, learning behaviour and some clinical disorders. Most of these functions in the physiologic and pathologic states have been identified to be mediated by H_1R and/or H_2R in the brain.

H_2R are coupled to Gs, adenylyl cyclase and protein kinase, which phosphorylates proteins and activates the transcription factor cyclic-AMP-response element (CRE)-binding protein (CREB). The direct action on neuronal membranes is usually excitatory or potentiates excitation (Francis *et al.,* 2007).

Unlike H_1R, H_2R are apparently not involved in the control of food intake, since injections of H_2R agonists do not decrease food intake (Sakata *et al.,* 1988). In addition, H_2R antagonists do not abolish the feeding suppressive effects of central histamine (Lecklin *et al.,* 1998; Morimoto *et al.,* 2001).

1.7.3 The H_3 receptor

The H_3R differs from the H_1 and H_2 receptors as it is located pre-synaptically (figure 1.7) and is expressed predominantly in the CNS, with high densities being found in the cerebral cortex, nucleus accumbens, striatum, olfactory tubercles and substantia nigra (Lovenberg *et al.*, 1999). H_1R and H_3R mRNAs are detected in a number of areas within the hypothalamus including the VMN and the ARC (Lovenberg *et al.,* 1999). Studies using selective H_3R ligand binding have shown a high density of H_3R in the hypothalamus, striatum and nucleus accumbens **(**Hussain *et al*., 2002). Although the highest densities of H_3R can be found in the CNS, they are also present in much lower levels within the PNS, for example, in the GI tract, the airways and the cardiovascular system (Celanire *et al.,* 2005). The histamine H_3R was discovered in 1983 by Arrang and co-workers (Arrang *et*

al., 1983) and later cloned (Lovenberg *et al.,* 1999). H_3R are located on the cell bodies and axon terminals of histamine-containing neurones, where they serve as autoreceptors to modulate histamine synthesis and release (Celanire *et al.,* 2005). H_3R also act as presynaptic heteroreceptors on the terminals of neurones that contain other transmitters, such as dopamine, serotonin, noradrenaline, GABA and acetylcholine, thus can also negatively regulate the release of a number of other neurotransmitters (Schlicker *et al.,* 1994) (figure 1.8). Since H_3R are located predominantly in the CNS, it has been suggested that H_3R mediate various CNS functions by modulating brain histaminergic tone and possibly by interacting with H_1R and H_2R. R-alpha-methylhistamine, a selective H_3R agonist, inhibits the release of endogenous brain histamine (Itoh *et al.,* 1998), while thioperamide, a selective inverse agonist, enhances histamine release (Itoh *et al.,* 1998, Itoh *et al.,* 1999).

H_3R is functionally linked to Gi/o proteins, thus negatively regulates intracellular cAMP levels (Lovenburg *et al.,* 1999, Chen *et al.,* 2003). The amino acid sequences of H_3Rs are highly conserved among humans, monkeys, rats, mice, and guinea pigs, thus suggesting that H_3Rs play critical roles in numerous species (Hancock *et al.,* 2003). Research has shown that several splice variants are present in humans, rats, mice and guinea pigs. Drutel *et al.* reported that rat H_3R splice variants (H_3A, B, and C) showed distinct expression patterns in the brain and were differentially coupled to Gi/o proteins when expressed in cultured cells (Drutel *et al.,* 2001). Observations suggest that the splicing variants might have distinct roles *in vivo,* but the full extent of which are still unknown. Although no major pharmacological differences have yet been noted for these isoforms using antagonists, agonists do show increased potencies for the short isoform (Wieland *et al.,* 2001). One important point to illustrate is that profound species differences in the antagonist pharmacology of the rat and human H_3Rs have been observed (Ligneau *et al.,* 2000; Lovenberg *et al.,* 2000; Yao *et al.,* 2003).

One of the remarkable features of H_3R is its high constitutive activity both *in vivo* and *in vitro* (Morisset *et al.,* 2000) (for a full and detailed description of H_3R constitutive activity see Introduction in Chapter 6 of this thesis). The high constitutive activity of H_3R may be critical for its regulatory function in the CNS (Morisset *et al.,* 2000, Schwartz *et al.,* 2003). Both native and heterologously expressed recombinant H_3Rs are constitutively active (Morisset *et al.,* 2000; Wieland *et al.,* 2001; Rouleau *et al.,* 2002), and as a result of this several previously characterized H_3R antagonists have subsequently been shown to be inverse agonists, including thioperamide.

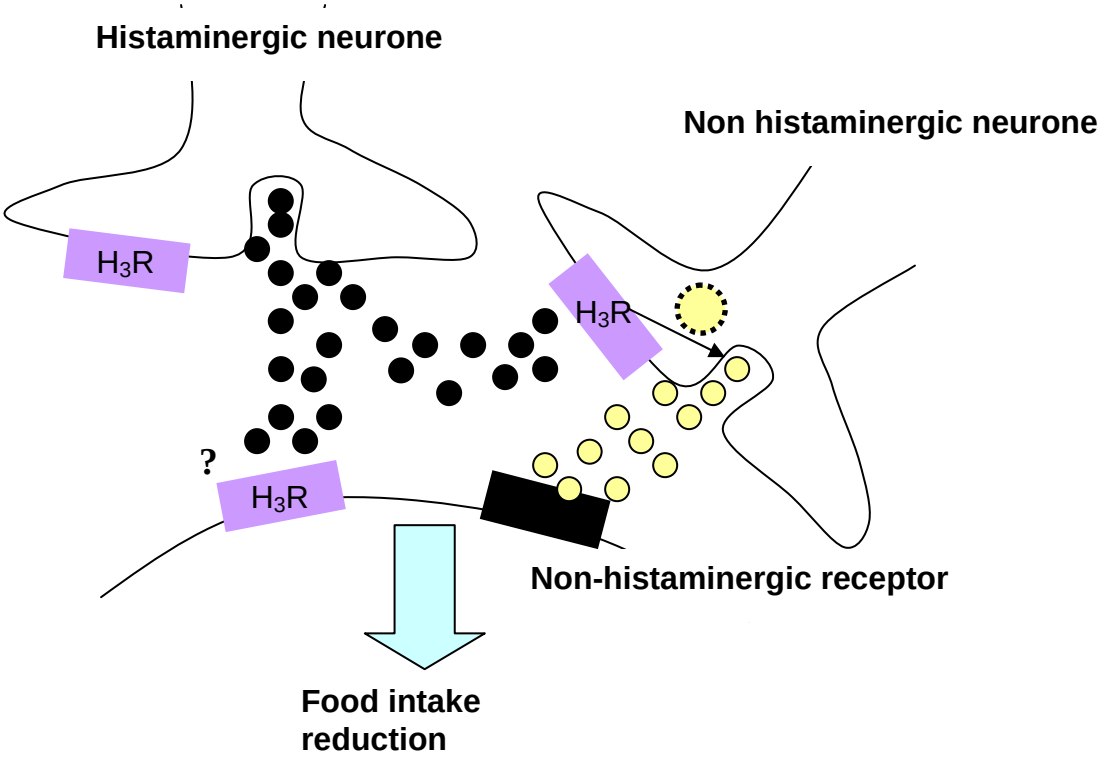

Figure 1.8.; Based on Yoshimoto *et al*., (2006) illustrating the regulation of H_3R on appetite. Released histamine acts on H_3R located on the presynaptic histamine terinal or on non-histaminergic neurones containing noradrenaline, dopamine, serotonin, glutamate, and GABA. Released histamine may also activate postsynaptic H_3R.

H_3R-specific ligands have been shown to have beneficial effects in animal models of obesity, epilepsy, and cognitive diseases such as Alzheimer's disease and attention deficit hyperactivity disorder (Hancock *et al.,* 2003; Passani *et al.,* 2004; Leurs *et al.,* 2005). Thus, H_3R antagonists/inverse agonists are considered as potential new therapeutics and are currently undergoing clinical trials (Celanire *et al.,* 2005).

The histaminergic system is well-documented for its involvement in reducing food intake through the histamine-induced activation of the H_1R, but the histamine H_3R also plays an important role in food intake and body weight regulation. H_3R agonists have been shown to increase food intake whereas the H_3R antagonists and inverse agonists appear to reduce feeding. The role of the H_3R in energy control are still being elucidated but due to its complexity it could be affecting feeding by acting via the H_1R or even by controlling the release of other neurotransmitters.

H_3R-KO mice display enhanced histamine release when compared with wild-type (WT) littermates, with increased histamine release being recorded in the forebrain, hypothalamus, thalamus, hippocampus, cerebellum and brainstem (Takahashi *et al.,* 2002). H_3R-KO mice are viable and fertile, but initially their body size does not differ significantly from WT littermates, and they show no overt abnormalities in the brain or other tissues. The key variation from the WT phenotype is that H_3R-KO mice display mild hyperphagia, decreased energy expenditure and late-onset obesity. The fact H_3R-KO mice become obese is a paradoxical finding as without the H_3R being present it would be expected that histamine release would cease to be negatively controlled and, thus, a increase in circulating endogenous histamine would be present, resulting in anorexic and even a lean mouse phenotype. Takahashi *et al.,* suggest it could be that a lack of H_3Rs alters neural circuitry during the development stage or sustained increases in histamine release may desensitize and/or down-regulate post-synaptic

histamine receptors (H_1R and H_2R), and hence attenuate net histaminergic tone, but the evidence for this has yet to be found (Takahashi *et al.,* 2002).

1.7.4 The H_4 receptor

The H_4R is a post-synaptic receptor and is coupled mainly to Gi/o proteins. In either stably or transiently transfected cells, H_4R activation leads to a pertussis-toxin-sensitive decrease in the forskolin-induced production of cAMP and the inhibition of downstream events such as CREB-dependent gene transcription in much the same way as the H_3R (Lui *et al.,* 2001 b). This observation indicates that the H_4R, like other histamine receptors expresses constitutively activity. The H_4R was cloned and found to be over 35% homologous to the H_3R (Lui *et al.,* 2001 b).

Research into the H_4R suggests it is involved principally in the immune system and expressed mainly during allergic reactions or as an inflammatory response (De Esch *et al.,* 2005, Nguyen *et al.,* 2001). The H_4R is expressed in the PNS, but to date there is no consistent observable evidence suggesting H_4R expression in the brain (Liu *et al.,* 2001 b, Welty *et al.,* 2009). O'Reilly *et al.* did report weak expression in the human brain, but expression has not been demonstrated in the brains of rat, mouse or guinea pig (O'Reilly *et al.,* 2002). Therefore, if these receptors are expressed in the brain they are expressed at minimal levels. No evidence has been found to suggest the H_4R has any role in the control of energy requisition or expenditure.

1.8 Histamine and feeding

Neuronal histamine is involved in the regulation of food intake (Morimoto *et al.,* 2001). Changes in endogenous histaminergic tone in the CNS have been

associated with genetic models of obesity (Machidori *et al.,* 1992), such as the *ob*/*ob* mouse (Hancock *et al.,* 2004). L-histidine, the precursor of histamine, decreases food intake when it is intraperitoneally (IP) injected (Orthen-Gambill N*.,* 1988, Sheiner *et al.,* 1985). The ICV infusion of histamine itself (Lecklin *et al.,* 1998) or the increase of endogenous histamine by the administration of metoprine, an inhibitor of histamine catabolic enzyme, suppresses food intake in rats (Lecklin 1995). α-FMH the specific suicide inhibitor of histidine HDC, inactivates HDC more specifically and potently than any other HDC inhibitors (Garbarg *et al.,* 1980). This inactivation of HDC is highly selective, so much so that α-FMH depletes neuronal histamine almost completely from the nerve terminals therefore increasing food intake (Garbarg *et al.,* 1980). Thus, pharmaceutical agents directed at HDC could help regulate the release and synthesis of histamine and, thus, be used as a drug to help control appetite.

H_1R and H_3R are both important for regulating feeding, and research suggests they have opposing affects on food intake. In both humans and rodents, treatment with a H_1R antagonist or H_3R agonist results in hyperphagia (Fukagawa *et al.,* 1989), and administration of a H_1R agonist or H_3R antagonist or inverse agonist leads to hypophagia (Attoub *et al.,* 2001, Takahashi *et al.,* 2002). These opposing effects on food intake are most simply explained by the fact that the H_1R is a postsynaptic receptor whereas the H_3R is a presynaptic autoreceptor. Evidence for histamine's role in food intake and appetite share the common theme that insufficient stimulation of post-synaptic CNS histamine H_1R causes increased food consumption and/or weight gain (Karlstedt *et al.,* 2001)**.** Studies have shown the ability of histamine H_3R antagonists or inverse agonists to promote weight loss or prevent weight gain (Hancock *et al*., 2004), with the presumed mechanism of action being the resulting enhanced release of histamine from histaminergic terminals, leading to the stimulation of postsynaptic histamine H_1R to reduce appetite. Thus, a presumed CNS mode of action is

likely, although findings from recent *in situ* hybridization histology indicating the prominent expression of histamine H_3R mRNA in brown adipose tissue (Karlstedt *et al.,* 2001) would also support a potential non-CNS role in regulating thermogenesis through peripheral sites of action (Hancock *et al*., 2004).

The H_1R has long been a target of interest in the regulation of food intake. ICV application of a H_1R agonist (2-(3-trifluoromethylpehenyl)histamine) potently suppresses food intake, whilst antagonism of hypothalamic H_1R's results in food intake (Sakata *et al.,* 1997; Han *et al.,* 2008). In addition, the H_3R is also important in the control of food intake. For example, antagonism of H_3R's in the hypothalamus reduces food intake and induces weight loss in diet-induced obese rodents, as well as rhesus monkeys and pigs (Hancock *et al.,* 2004; Malmlof *et al.,* 2005; Malmlof *et al.,* 2007), whilst the H_3R agonist, imetit enhances feeding in rats (Chiba *et al.,* 2009). Furthermore, the H_3R's localisation in the hypothalamus and NTS is supportive of its potential role in the regulation of food intake (Poole *et al.,* 2008). The H_3R has a distinct role in numerous appetite signaling pathways. For example, the H_3R inverse agonist, thioperamide, inhibits food intake induced by the potent orexigens NPY and PYY (Itoh *et al.,* 1999), whilst H_3R agonists, R-α-methyl-histamine (α-MH) and imetit, decrease bombesin-induced satiety in rats (Kent *et al.,* 1997). In addition, H_3R activation reduces the anorexigenic effects of amylin in fasted rats (Lutz *et al.,* 1996). A study by Attoub *et al.* found that α-FMH dose-dependently inhibited the satiating effects of CCK, whilst thioperamide enhanced CCK-induced satiety. They also found that pre-treatment with pyrilamine, a post-synaptic H_1R antagonist, inhibited CCK-induced satiety (Attoub *et al.,* 2001), indicating that whilst the H_3R controls pre-synaptic histamine concentrations, CCK-induced satiation is also dependent upon the post-synaptic activation of the H_1R.

In mice and rats, the activity level of the histaminergic system is relatively high at night and low in the day (Mochizuki *et al.,* 1992). However, these animals

eat at night for the most part although at this time the histamine activity is high. Sakata *et al.,* showed that H_1R (Sakata *et al.,* 1988) and α-FMH (Sakata *et al.,* 1990) antagonism induced food intake when they were modulated soon after lights on, but their effects were attenuated in the dark phase. In addition, they also showed that thioperamide reduces food intake in the dark (Sakata *et al.,* 1990). If this is the case, the abilities of these drugs to affect food intake inversely correlates with the histamine level in both the light and dark phase (Morimoto *et al.,* 2001).

1.9 Histamine and its interactions with other transmitters and peptides

As mentioned previously, the histamine H_3R can act as a heteroreceptor and it is well documented that these receptors are present on the neurones of a number of other neurotransmitters. Thus, the H_3R not only regulates the release of histamine itself but also of other transmitters (Schlicker *et al.,* 1994). Research indicates that the H_3R's modulate the release of dopamine, acetylcholine, serotonin, GABA and noradrenaline in the brain (Schlicker *et al.,* 1993). Also, secretion of these neurotransmitters is inhibited by the activation of H_3R (Arrang *et al.,* 1995), whilst H_3R antagonism disinhibits their release.

The first effective anti-obesity drugs (fenfluramine, sibutramine) that were developed to inhibit food intake and limit weight gain acted upon the synaptic availability of dopamine, acetylcholine, serotonin, GABA and noradrenaline. The clinical efficacy of these drugs can be attributed to their ability to enhance serotonin availability (Tallett *et al.,* 2009), and it has been reported that the H_3 heteroreceptors can inhibit serotonin release resulting in an increase in food intake (Cole *et al.,* 1998). Also, the anti-histamines, cyproheptadine and promethazine, are non-selective serotonin receptor antagonists, as well as acting as histamine receptor blockers that induce food-intake and weight gain in humans

and rats (Yoshimatsu *et al.,* 2002). H_3 heteroreceptor activity may also play a role in atypical antipsychotic-induced weight gain. Antagonism of the H_3 heteroreceptor has been shown to disinhibit neurotransmitter release (Arrang *et al.,* 1995), which in the case of H_3 heteroreceptors located on noradrenaline and acetylcholine neurones, may account for the side-effect of atypical antipsychotic-induced food intake, as both these neurotransmitters can increase food intake (Pratt *et al.,* 2009).

Histaminergic neurones project fibres to almost all brain areas, albeit to a lower extent than the hypothalamus. The hippocampus and the nucleus accumbens both receive innervations form histaminergic neurons and both exert some physiological control of appetite (Hussain *et al*., 2002). High densities of H_1R and H_3R have both been found in the striatum (Drutel *et al.,* 2001, Pillot *et al.,* 2002). The analysis of histamine receptor distribution has shown that the nucleus accumbens also contains high levels of H_1R and H_3R, and comparably lower levels of H_2R. Studies have indicated functional interaction between histaminergic and dopamine neurotransmission, with histaminergic function regulating at least in part dopamine activity in the forebrain (Galosi *et al.,* 2001, Hussain *et al.,* 2002). Shlicker *et al.* found that histamine can inhibit striatal dopamine release and synthesis via presynaptic H_3R (Shlicker *et al.* 1993). However, the complete mechanism by which histamine exerts its effects on dopamine activity is not clear. Prast *et al.* suggested histamine may be acting presynaptically through histamine receptors located on dopamine terminals in the nucleus accumbens (Prast *et al.,* 1999). Alternatively, Galosi *et al.* propose histamine may act on the output neurones of the nucleus accumbens, which via downstream projections may indirectly stimulate dopaminergic cells in the ventral tegmental area, resulting in dopamine release in the nucleus accumbens. Histamine can also interfere with the uptake of dopamine (Galosi *et al.,* 2001), which could also lead to increased levels of extracellular dopamine. It may also be possible that histamine affects

dopamine release via acting on other inputs to the nucleus accumbens (Prast *et al.,* 1999).

As the histaminergic cell bodies are located only within the TM of the posterior hypothalamus (Watanabe *et al.,* 1984), it is possible the signal inputs to the TM greatly influence histaminergic function Morphological evidence has suggested some of the appetite-stimulating peptides affect the histaminergic system within the TM itself (Watanabe *et al.,* 1984). Neurones containing orexin-A and -B densely innervate the TM (Peyron *et al.,* 1998), and both of the orexin receptors are expressed in the TM (Eriksson *et al.,* 2001). NPY neurones have been shown to form synapses with the histaminergic cells directly in the TM (Tomaszuk *et al.,* 1996). Guan *et al.* reported strong expression of mRNA for the growth hormone-secretagogue receptor, for which ghrelin is an endogenous ligand, in the TM (Guan *et al.* 1997). Also, Nakazato *et al.* reported that ICV injections of ghrelin induced the induction of c-*fos* expression in the TM (Nakazato *et al.* 2001). Thus, ghrelin could affect the histaminergic system directly in the TM. These observations indicate a functional relationship between these peptides and the histaminergic system (Ishizuka *et al.,* 2006).

Neuronal histamine plays an important role in leptin signaling in the hypothalamus, which controls feeding behaviour and energy metabolism. The central administration of leptin increases histamine turnover (Yoshimatsu H., 2008). Leptin-induced suppression of feeding is attenuated in histamine-depleted rats using α-FMH, HDC and in histamine H_1R-KO mice (Masaki *et al.,* 2001). All these findings indicate neuronal histamine and the H_1R could be mediating the suppressive effect leptin has on food intake (Morimoto *et al.,* 1999).

NPY has been demonstrated to potently increase food intake in both WT and H_1R-KO mice, however the increase in food intake observed in H_1R-KO mice was much more significant than that seen in WT animals (Ishizuka *et al.,* 2006). Ishizuka *et al.* found NPY increases histamine release although this was very

short lived (Ishizuka *et al.,* 2006). Further evidence of NPY and histamine working together in the control of food intake is that NPY afferents form direct synaptic contacts with the histaminergic cell body, and mRNA of NPY Y1 receptors, which has been implicated in feeding (Kanatani *et al.,* 2000), is expressed in the TM (Kishi *et al.,* 2005). These observations suggest that NPY could act on the histaminergic system directly in the TM via the NPY Y1 receptors. Toftegaard *et al.* have previously shown that the suppression of neuronal histamine synthesis by α-FMH stimulates NPY mRNA expression suggesting histamine exerts an inhibitory effect on NPY synthesis (Toftegaard *et al.,* 2003). Therefore, NPY may inversely affect histamine synthesis and form a feedback loop with the histaminergic system in the regulation of food intake.

1.10 Summary

Obesity is clearly a worldwide problem in most developed countries and is costing health services around the world millions in treating obesity-related illnesses. Thus, it is paramount that treatments are developed which help tackle this growing pandemic. Scientific research has advanced over the last few years and gained vital knowledge and understanding of the central mechanisms and pathways involved in regulating food intake and the control of energy balance. One widely accepted discovery is the central role the hypothalamus has in regulating energy balance and controlling feeding.

Histamine is an important neurotransmitter within the CNS that has been associated with the regulation of appetite. Histamine has been shown by numerous research groups to act as a potent anorexigenic agent (Doi *et al.,* 1994, Endou *et al.,* 2001, Lecklin *et al.,* 1998, Lecklin *et al.,* 1995). The histaminergic system acts via the H_1R or H_3R to exert its effects on feeding and, thus, these receptors could act as possible targets in the development of therapies to battle obesity. These receptors have opposing effects on feeding in that H_1R agonists acts as potent anorexigens and H_3R agonists act as powerful orexigens. Although the H_1R has been more widely investigated as a possible target for anti-obesity drug development the fact these receptors are widely distributed and are involved in many functions, agonist drugs which target them can cause numerous adverse effects. For example, H_1R agonists would have profound effects on the immune system (note anti-histamines acting on the H_1R are used to combat hayfever and other allergic reactions). Instead, because H_3R negatively regulate the release of histamine mainly in the brain, potentially to suppress appetite through the activation of only central H_1R targets, pharmaceutical organisations have turned their focus onto H_3R-based antagonists and/or inverse agonists, which can be given systemically but will have their

effects only centrally. To address the therapeutic potential of H_3R ligands as anti-obesity drugs, several groups have reported the pharmacological profiles of H_3R inverse agonists in animal studies.

Although H_3R inverse agonists have been seen to potently reduce food intake in a number of species (Lecklin *et al.,* 1998), research groups have shown varying degrees of their efficacy at changing food intake (Itoh *et al.,* 1998, Sindelar *et al*., 2005). Therefore, although some reports have suggested the therapeutic potential of H_3R inverse agonists, their anti-obesity effects remain controversial. For example, thioperamide, an imidazole-containing H_3R inverse agonist, has been shown to suppress food consumption in rats and mice (Lecklin *et al.,* 1998). However, other studies have shown thioperamide to have no affect on food intake either in satiated or in fasted rats (Itoh *et al.,* 1998). Sindelar *et al*., demonstrated that both IP and oral administration of thioperamide enhanced histamine release in the brain, while only IP administration caused significant reductions in food intake (Sindelar *et al*., 2004). Furthermore, one group found that thioperamide even increased food intake when administered to mice (Yoshimoto *et al*., 2006). The area of activation of these compounds has not been fully elucidated and the specificity of these drugs is not fully known. Thus, it is apparent that further investigation into the histaminergic system and in particular the H_3R is required.

1.11 PhD Aims

My main aims with this PhD were to investigate and determine histamine's hypophagic affect on feeding and to determine whether this system could be manipulated for the development of potential therapeutic agents that are anti-obesity targets.

I aimed to firstly determine the effects histamine had on rodent feeding when administered centrally. Once an anorexigenic effect had been elucidated we aimed to investigate whether H_3R-based drugs, including a H_3R agonist and two H_3R inverse agonists could also alter food intake. Our next aim was to investigate where histamine and these H_3R drugs were acting within the rodent brain and in particular whether the known 'feeding' or 'satiety' centres are involved. Once the areas in which histamine is acting had been established my next step was to investigate what effects histamine and histamine-based drugs have on neuronal firing within the VMN, a hypothalamic area known to be pivotal in the regulation of food intake. We then investigated whether histamine was exerting its effects within the VMN via a H_1R or a H_3R to determine the populations of histamine receptors present in this area. My penultimate aim was to ensure the effects of the H_3R drugs being tested were acting specifically at the H_3 receptor we co-applied a neutral H_3R agonist with an H_3R agonist or H_3R inverse agonist, to ensure the any effects these drugs had on feeding or neuronal firing were receptor specific. My final aim was to establish the same populations of VMN neurones responding to histamine are also glucose responsive.

Chapter 2:

In vivo effects of histamine and H_3R drugs on food intake

2.1 Introduction

2.1.1 Pharmaceutical development of histaminergic agents

Extensive pharmacological experiments have demonstrated that the brain's histamine system plays critical roles in the regulation of feeding and energy expenditure and, thus, this system could be manipulated for the pharmaceutical control of appetite and body weight. Behavioural studies have revealed histamine suppresses food intake when administered centrally or peripherally (Doi *et al.,* 1994, Endou *et al.,* 2001; Lecklin *et al.,* 1998; Lecklin *et al.,* 1995), therefore, Orthen-Gambill *et al.* (1992) suggested the removal of histamine's inhibitory effect might in turn stimulate feeding. They found that if histamine levels were decreased using α-FMH, which is a potent and irreversible inhibitor of HDC, an increase in both food intake and body weight is seen in rodents. This inactivation of HDC in histamine-containing nerve terminals is highly potent and selective; so much so, that FMH depletes neuronal histamine almost completely from the nerve terminals (Garbarg *et al.,* 1980). Hence, pharmaceutical agents directed at HDC could help regulate the release and synthesis of histamine and be used as a drug to help control appetite. However, a problem arises since such drugs would affect all histaminergic systems in the body.

An alternative would be to target specific histamine receptors. H_1R and H_3R are both important for regulating feeding. Their activity has opposing effects on food intake. H_1R agonists and H_3R antagonists decrease food intake, whereas H_1R antagonists and H_3R agonists increase food intake in rodents (Takahashi *et al.,* 2002). This is most simply explained by the fact that the H_1R is a postsynaptic receptor, whereas the H_3R is normally a presynaptic autoreceptor. In both humans and rodents, treatment with an H_1R antagonist results in hyperphagia

(Fukagawa *et al.,* 1989), whereas administration of H_3R antagonists leads to hypophagia (Attoub *et al.,* 2001).

The fact that drugs with H_1R-blocking properties stimulate food consumption in humans as well as in animals clearly indicates the critical role of endogenous histamine and H_1R in the regulation of feeding behaviour (Fukagawa *et al.,* 1989). However, H_1R are widely distributed and are involved in many functions, so agonist drugs which target them can cause numerous adverse effects. For example, H_1R agonists would have profound effects on the immune system (note anti-histamines acting on the H_1R are used to combat hayfever and other allergic reactions). Instead, because H_3R negatively regulate the release of histamine mainly in the brain, potentially to suppress appetite through the activation of only central H_1R targets, pharmaceutical organisations have turned their focus onto H_3R based antagonists and/or inverse agonists, which can be given systemically but will have their effects only centrally.

H_3R antagonists have been proposed as drugs for the treatment of several CNS disorders such as attention-deficit hyperactivity disorder (Onodera *et al.,* 1998), Alzheimer's disease (Passani *et al.,* 2000), epilepsy (Yokoyama *et al.,* 1994), schizophrenia (Schlicker *et al.,* 1996) and obesity (Takahashi *et al.,* 2002), whereas the therapeutic potential of H_3R agonists has been shown for myocardial ischemia (Levi *et al.,* 2000), inflammatory diseases (McLeod *et al.,* 1998), gastric acid-related diseases (Bertaccini *et al.,* 1991), and migraine and sleep disorders (Mignot *et al.,* 2002).

With the cloning of the H_3R and the discovery of the high degree of constitutive activity of this receptor, many H_3R antagonists have been subsequently reclassified as inverse agonists because of their ability to reverse basal H_3R activity. Since H_3Rs may inhibit neurotransmitter release in the absence of endogenous histamine because of their inherent constitutive activity (Morisset *et al.,* 2000), compounds that demonstrate greater inverse agonist efficacy may

cause greater enhancement of neurotransmitter release and thus could pose greater therapeutic effects. To address the therapeutic potential of H_3R ligands as anti-obesity drugs, several groups have reported the pharmacological profiles of H_3R inverse agonists in animal studies. Because H_3Rs negatively regulate the release of histamine in the brain, H_3R inverse agonists are believed to suppress appetite through the activation of H_1Rs in the post-synaptic areas. Although reports have suggested the therapeutic potential of H_3R inverse agonists, their anti-obesity effects still remain controversial. For example, thioperamide, an imidazole-containing H_3R inverse agonist, increases histamine neurones activity *in vivo* by removing the normal feedback autoinhibitory control system of both histamine synthesis and release at the level of histamine nerve terminals (Arrang *et al.,* 1987). Thioperamide has been shown to suppress food consumption in a number of animal models, for example Jethwa and colleagues showed that in the seasonal hamster administering H_3R inverse agonists in the lean state reduced food intake (Jethwa *et al.,* 2009). Whilst other studies have shown thioperamide to have no effect on food intake either in satiated or in fasted rats (Itoh *et al.,* 1998, Lecklin *et al*., 1998, Sakata *et al.,* 1990). Sindelar *et al*. (2004) demonstrated that both intraperitoneal (IP) and oral (PO) administration of thioperamide enhanced histamine release in the brain, while only IP administration caused significant reductions in food intake (Sindelar *et al*., 2004). Most recently, one influential report witnessed the opposing effects of thioperamide in murine studies (Yoshimoto *et al*, 2006). Here, the group showed that the H_3R inverse agonist thioperamide increased rather than decreased food intake, which opposes the findings of most research groups investigating the actions of these histamine-based drugs, but illustrates a need for further research. In addition, several reports have demonstrated the possibility that the imidazole moiety of thioperamide may be responsible for toxicity through its interaction with P450 proteins (Yang *et al.,* 2002; Onderwater *et al.,* 1998), thus

suggesting the possibility that imidazole-containing H_3R drugs may exert non-selective adverse effects leading to reduced feeding. In order to address these concerns, several pharmaceutical companies have developed non-imidazole H_3R inverse agonists (Tokita *et al.,* 2006). Among these, Hancock and colleagues reported that A-331440 potently suppresses feeding and body weight gain in diet-induced obese mice (Hancock *et al.,* 2004). However, ABT-239, another structurally distinct H_3R inverse agonist, showed no anti-obesity activity in the same animal model (Tokita *et al.,* 2006). Wulff *et al.* reported that the H_3R inverse agonists by Novo Nordisk, NNC-0038-1049 (NNC1049) and NNC-0038-1202 (NNC1202), reduced body weight in diet-induced obese rats (Wulff *et al.,* 2002). Furthermore, NNC1202 reduced food consumption and body-weight gain in pigs and obese rhesus monkeys. These varying pharmacological observations demonstrate that H_3R inverse agonist may possess anti-obesity activity but that their efficacy may differ among species (Tokita *et al.,* 2006). Many observations highlight potential and as of yet, unknown off-target activity of specific compounds that might contribute to their anorectic effects. Although the brain histamine system is a highly conserved system among species, it is possible that this system plays slightly different physiological roles. Oishi *et al.* reported that histamine content and turnover in several brain areas differ between mice and rats (Oishi *et al.,* 1983), thus supporting the notion of species related differences in H_3R ligand functions. Splicing variants, which differ slightly amongst species, would play different roles within the CNS, interacting differently with pharmacological reagents and therefore resulting in distinct outcomes in each species. Thus, further investigation of the use of H_3R compounds as potential anti-obesity therapies is required.

Imetit was initially characterized as a weak partial H_2R agonist (Ganellin R., 1981), but was later identified in various *in vitro* and *in vivo* tests as an extremely potent and selective H_3R agonist (Garbarg *et al.,* 1992). As an H_3R agonist it

would be expected that imetit would increase food intake by reducing histamine synthesis and release. This has been shown as the case for a number of research groups (Garbarg *et al.,* 1992; Leurs *et al*., 2005; Hancock *et al*., 2004) who all demonstrated imetit produces a increase in food intake in rodent studies. However, despite disagreeing with this published material, Yoshimoto *et al*, reported that imetit reduced, rather than increased food intake in mice and continual injections even resulted in a body weight reduction (Yoshimoto *et al*., 2006). Again, these data emphasise the confused literature and show the need for further studies to be carried out to determine the exact actions of H_3R drugs on food intake.

Additionally, in mice and rats, the activity level of the histaminergic system is relatively high at night and low in the day (Mochizuki *et al.,* 1992). However, these animals eat mainly at night when the histamine activity is high. Sakata *et al.,* showed that H_1R antagonism (Sakata *et al.,* 1988) and α-FMH (Sakata *et al.,* 1990) induced food intake when they were injected soon after lights on, but their effects were attenuated in the dark phase. In addition, they also showed that thioperamide reduces food intake in the dark (Sakata *et al.,* 1990). If this is the case, the abilities of these drugs to affect food intake inversely correlates with the histamine level in both the light and dark phase (Morimoto *et al.,* 2001).

2.2 Aims and Objectives

2.2.1 Does histamine reduce food intake when administered centrally?

Histamine has many roles in mammalian physiology, one of which is its powerful effect on appetite. The hypothalamus is integral in appetite control and in order to confirm histamine's actions in our own models and to lay the

foundation for investigating receptor-selective drugs, we carried out a baseline study to determine the central action of histamine on food intake.

2.2.2 What effects does the H_3R receptor agonist have on food intake?

Controversy over the effects of H_3R agonist drugs has been raised, thus, we aimed to determine the effect of the H_3R agonist, imetit, on food consumption in the adult rat when administered both centrally and systemically.

2.2.3 What are the effects on food intake of H_3R inverse agonists?

This same controversy over the effectiveness of H_3R agonists also applies to the H_3R inverse agonists. Thus, we ascertained the effects of the H_3R inverse agonists, thioperamide and the coded compound from Novo Nordisk, NN1202, on food consumption in the adult rat to help establish if these compounds could possibly be used pharmacologically to reduce food intake.

2.2.4 Do the H_3R selective drugs have indirect, adverse effects on food intake?

Our last objective was to establish if the H_3R drugs being considered altered only the feeding behaviour, or if other adverse responses, such as malaise or sedation, occurred resulting in secondary actions on food consumption. In order to determine this, we studied the behavioural satiety sequence of different experimental groups.

Normally, food intake is controlled by appetitive processes such as hunger or satiation. However, many compounds can alter food intake when administered exogenously. Although to date it is not fully understood how histaminergic drugs affect appetite and alter food intake, one explanation is that they are acting upon a specific stage of the natural process where animals match their food intake to nutritional requirements (Blundell *et al.,* 1985). A reduction in food intake instead

could be due to non-specific, non-physiological interference of eating behaviours, such as a reduction in motor function or malaise. Thus, one question is whether drug-induced alterations in food consumption actually reflect the natural process of satiation (Blundell *et al.,* 1975). To determine this, more data is required than simply a change in food consumption. Thus, additionally we can analyse changes in the structure of feeding behaviour. The examination of the structure of behaviour can be used to identify adaptive responses to "natural" processes or altered responses brought about by pathological, physiological or environmental conditions, such as administering drugs. Therefore, the structure of behaviour can be used to determine whether the effect of a drug on food intake is mediated by a natural process or a pathological condition (Blundell *et al.,* 1985).

2.2.5 Do imetit and thioperamide have similar effects on food intake in mice?

The paper published by Yoshimoto *et al.* (2006) suggested the H_3R agonist imetit could decrease the food intake in mice, that prolonged administration could even cause a loss of body weight, and that the H_3R inverse agonist thioperamide had the opposite effect to increase food consumption. This paper was very influential because, until then, all published research into the effects of these drugs on food intake suggested the opposite, with imetit causing hyperphagia and thioperamide having an anorectic effect. At face value, because histamine itself is anorexic, the results from Yoshimoto and colleagues (2006) are unusual since imetit should decrease endogenous histamine release, while thioperamide should increase it.

2.3 Methods

2.3.1 Animals

Male Sprague-Dawley rats (225 - 275 g) and male CD1 mice (7 weeks old; both Charles-River, UK) were kept in a 12 h/ 12 h light-dark cycle (lights on 08.00-20.00) within The University of Manchester animal facility. All animals were maintained in the facility for at least one week prior to the start of the experiment. The animals were housed in a temperature-controlled room (~22 ºC ± 1) with a relative air humidity of 40-60%. They had free access to food (Beekay, UK) and water.

These and all other experiments conformed with the Home Office (Animals) Procedures Act (1986) and local ethical review.

2.3.2 Compounds

Compounds were administered via injection into the lateral cerebral ventricle for intracerebroventricular administration (ICV) and into the intraperitoneal cavity (IP) for the systemically-injected animals. Histamine (Sigma, Sigma-Aldrich Company Ltd, Dorset) and was diluted in isotonic saline to a concentration of 200 nmol immediately prior to use. Thioperamide (Sigma) was diluted in isotonic saline to the required amount for the ICV (100, 200, 400 nmol per rat) and IP (0.5, 1, 2 mg/kg body weight) injections. Imetit (Sigma) was diluted with isotonic saline to the required amount for the ICV (10, 50, 100 nmole per rat) and IP (2.5, 5, 10 mg/kg body weight) injections. NNC1202 was donated by Novo Nordisk and was diluted in isotonic saline to the necessary amounts for the ICV (100, 200, 400 nmole per rat) and IP (10, 20, 30 mg/kg body weight) injections.

For the murine experiments drugs were diluted in the same manner as the rodent IP studies, but the following amounts were used: thioperamide and imetit

were injected at 10 or 20 mg/kg body weight (in a volume of 1 ml/kg of body weight).

2.3.3 Intracerebroventricular cannulation

Rats were anaesthetised with 2 % isofluorane in O_2 at 1 l/min. Once the head had been shaved, rats were placed securely in the stereotaxic apparatus (Stoelting, Illinois, USA). A 2-3 cm incision was made along the midline of the cranium, exposing the skull. All connective tissue was displaced from the cranial surface before the guide cannula was positioned 0.8 mm posterior and 1.5 mm lateral to bregma (co-ordinates determined according to Paxinos and Watson 1986). A 21-gauge guide cannula was implanted 3 mm into the brain at this co-ordinate through a 1.5 mm-diameter hole drilled in the cranium so it inserted into the lateral ventricle. The guide cannula was fixed to the skull with acrylic dental cement (Simplex Rapide; Austental Dental, UK) adhered to two jeweller's screws (4 mm long x 2 mm diameter) positioned anterior and posterior to the guide cannula. The dental cement was allowed to dry and the skin around the cannula and the surrounding skin was then sutured (size 3/0 mersilk, Ethicon, Johnson and Johnson International, USA). For post-operative analgesia, rats were injected with 10 µl/kg buprenophrine (Vetergesic, Reckitt Benckiser Healthcare, Hull, UK), and a bolus of saline to aid recovery. Animals were allowed to recover and then maintained for one week post surgery. During this time rats were given food and water *ad libitum*. A cannula made from the tip of a 23-gauge needle was used to inject the drug or vehicle when inserted through the guide cannula to a depth of 10.5 mm from the surface of the skull which allowed the drug to be injected directly into the lateral ventricle.

2.3.4 The effect of histaminergic drugs on food intake

The control groups in the feeding studies show some variation between the absolute amounts of food eaten. Rats naturally show much variation in food consumption from experiment to experiment, which is probably dependent on individual variations, the exact time of day that an experiment was begun, the amount of food eaten by different groups in the period immediately before each experiment, as well as the immediate pre-history of handling and husbandry. The daytime feeding experiments all began between the hours of 8:00 am and 10:00 am. Although all rats are managed in as similar a way as possible, variations in the daily maintenance of stocks by the animal facility staff are difficult to control. Variations between experiments are relatively small, but to ensure statistical relevance to all experiments, the drug-injected experimental groups and the control groups are housed and maintained, as well as treated, in exactly the same way. That is every experiment has its own internal controls.

Rats were caged singly and fasted overnight prior to both ICV and IP injections. On the day of the experiment for central injections, rats were administered 2 µl of the drug being tested (amounts used above) and control rats received 2 µl saline ICV. After the injection, the cannula was checked to ensure reflux did not occur. For both the rat and mouse systemic injections, on the day of the experiment, animals were administered 1ml/kg (body weight) of the drug being tested (amounts used above) and controls received 1 ml/kg saline IP. Pre-weighed food was returned to the animal 2 minutes after receiving the injections. Food consumption was measured at 1, 2, 4, 8, and 24 h intervals post-injection for the rodent studies and 1, 2, 4, 12 and 24 h for the murine studies.

2.3.5 Behavioural satiety sequence (BSS)

Each experiment was performed at the same time every day to avoid any circadian variation. Rats were housed singly, two days before the experiment, in transparent observation cages. Food was removed 2 hours before the experiment began to ensure no pre-feeding occurred. Animals (n = 8 per treatment) received a IP injection of isotonic saline, imetit (10 mg/kg body weight), thioperamide (2 mg/kg) or NNC1202 (30 mg/kg) at 20:00 h on different days, so each animal received one injection of each compound or vehicle over a two-week period. A minimum of 3 days were left between each experiment so animals had recovered from their last drug treatment. After the drug was administered, rats were given a pre-weighed amount of food. Behavioural observations began 90 seconds later. The animals were scored every 30 sec for 90 minutes with the behaviour of each animal at that instant in time being noted. Their behaviour was scored as one of the following: feeding, drinking, grooming, resting, inactive or active (see table 2.1). The data were collated into 5-minute periods or bins and expressed as the mean percentage of total behaviour. The percentage of time the animals spent exhibiting each individual behaviour was analysed for all drug or vehicle groups and collated into a graph. The overall food eaten for each animal was taken after the 90-minute observational period was over.

Resting	Sitting or lying in a resting position or asleep.
Inactive	Not moving or exploring but not lying in resting position and still fully awake.
Active	Walking around cage, exploring or circling. Movements involving all four limbs.
Grooming	Scratching, licking or biting of the coat, whiskers, feet or genitals. Biting of the tail.
Drinking	Licking the water bottle.
Feeding	Biting, gnawing or swallowing food from dish or from front paws.

Table 2.1.; This table was adapted from Halford and Blundell 1993 and illustrates the six behavioural categories used for behavioural analysis when carrying out the behavioural satiety sequence (BSS) experiments.

2.3.6 Statistical anaylsis

All data are presented as mean ± standard error of mean (S.E.M.). Data from feeding and the BSS studies were analysed using an unpaired t test, one-way analysis of variance (ANOVA) with Bonferonni *post hoc* test or a two-way ANOVA with repeated measures were carried out depending on the experiment. Significance was taken at $P < 0.05$.

2.4 Results

2.4.1 Histaminergic effects on food intake in rats

Injection of histamine ICV (200 nmol in 2 µl) caused a significant suppression of food intake in rats ($P < 0.05$) (figure 2.1). It was observed that one hour after administration, rats that received histamine had eaten only 0.9 ± 0.3 of food, whereas those that received saline had eaten 6.2 ± 0.4 g ($P < 0.005$), and a statistically significant difference in cumulative intake remained until 8 hours after ICV injection ($P < 0.05$). At 24 hours post-injection the cumulative food eaten by the two groups was no longer significantly different: histamine injected rats had eaten 25.2 ± 0.8 g of food and rats that had received saline had eaten 26.1 ± 1.9 g.

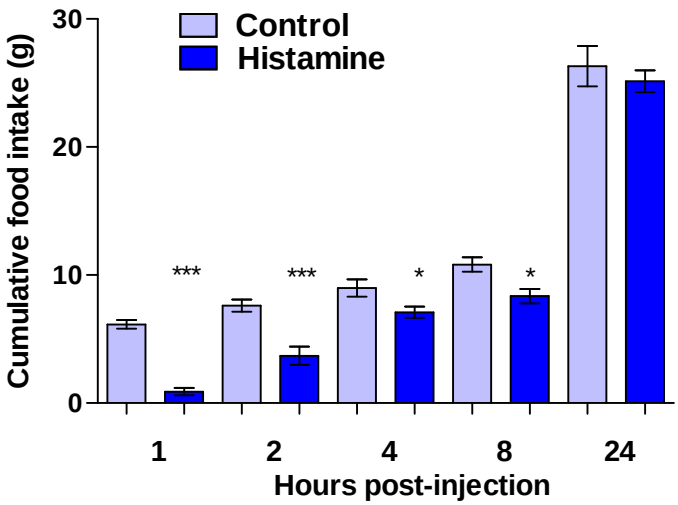

Figure 2.1.; Food consumption measured at 1, 2, 4, 8 and 24 hours after ICV injection with histamine (200 nmol, n = 11) or saline (n = 9). Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: *** $P < 0.005$, * $P < 0.05$.

2.4.2 H_3R agonist effects on food intake in rats

We carried out ICV injections using imetit (H_3R agonist) at varying concentrations (100, 50 and 10 nmol) to determine a dose-response curve (figure 2.2). We found that giving imetit at a concentration of 10 nmol was not sufficient enough to produce a significant increase in food intake. Both 50 nmol and 100 nmol of imetit caused rats to significantly increase their food consumption 1 hour after the initial injection (figures 2.2 and 2.3).

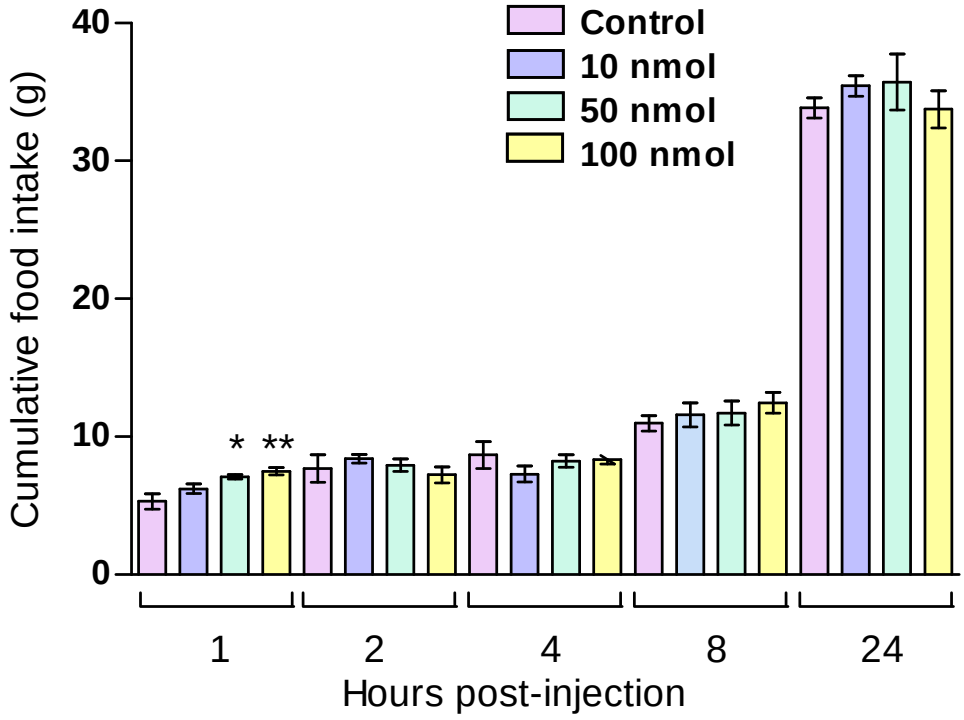

Figure 2.2.; Food consumption measured at 1, 2, 4, 8 and 24 hours after ICV injection with imetit (10, 50, & 100 nmol) or saline, in 2 µl. n = 7 for saline and 100 nmol imetit groups and n = 6 for 10 nmol and 50 nmol imetit drug groups. Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: ** *P < 0.01,* * *P < 0.05*.

The group that received 50 nmol of imetit ate 7.08 ± 0.19 g 1 hour after receiving the ICV injection whereas control animals ate 5.30 ± 0.56 g. This showed a significant increase in food intake ($P < 0.05$). This increase in food intake was not a long-term effect as by 2 hour the significance was lost, with the 50 nmol imetit group having cumulatively eaten 7.79 ± 0.52 g and the control groups having cumulatively eaten 7.67 ± 0.99 g (figure 2.3). Out of the concentrations tested, we found that giving imetit at a concentration of 100 nmol gave the most significant results. An ICV injection of 100 nmol imetit caused a significant, though short-term, increase in food intake in rats (figure 2.3). It was observed that one hour after treatment, rats that received imetit had eaten 7.5 ± 1.3 g of food whereas those that received saline ICV had eaten 5.3 ± 1.9 g ($P < 0.01$; figure 2.3). The significance was lost between 1 and 2 hours post injection with rats receiving ICV imetit injections having a cumulative food intake of 8.4 ± 1.2 g compared with the saline-treated group 7.67 ± 0.99 g (figure 2.3).

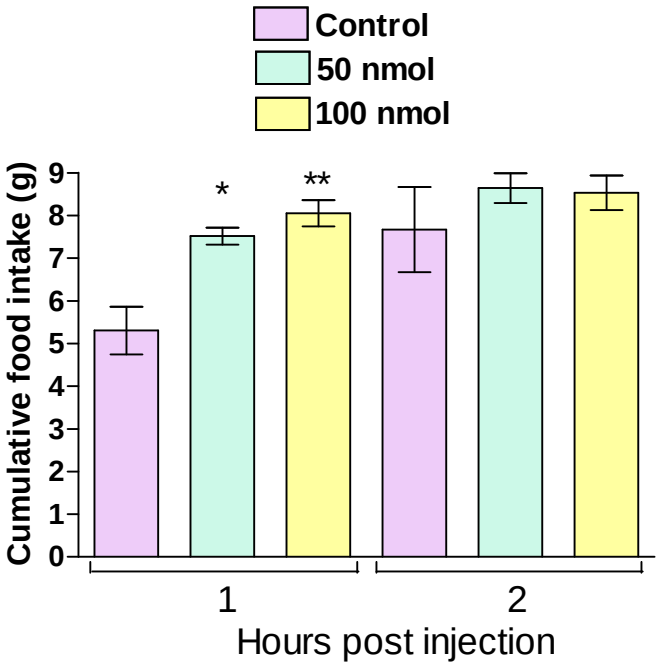

Figure 2.3.; Food consumption measured at 1 & 2 hours after ICV injection with imetit at 50 & 100 nmol or saline, in 2 µl. n = 7 for saline and 100 nmol imetit groups and n = 6 for 50 nmol imetit drug group. Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: ** *P < 0.01, * P < 0.05*.

We also carried out a dose response curve for imetit (H_3R agonist) given IP at varying amounts (10, 5 and 2.5 mg/kg, see figure 2.4). We found that imetit when given at 2.5 mg/kg and 5 mg/kg there was no significant change in food intake, although the lower amount (2.5 mg/kg) did surprisingly show some trend towards reducing food intake, but this did not enter significance. Alternatively, when we administered imetit IP at 10 mg/kg a significant increase in food intake in rats was observed (figures 2.4 and 2.5).

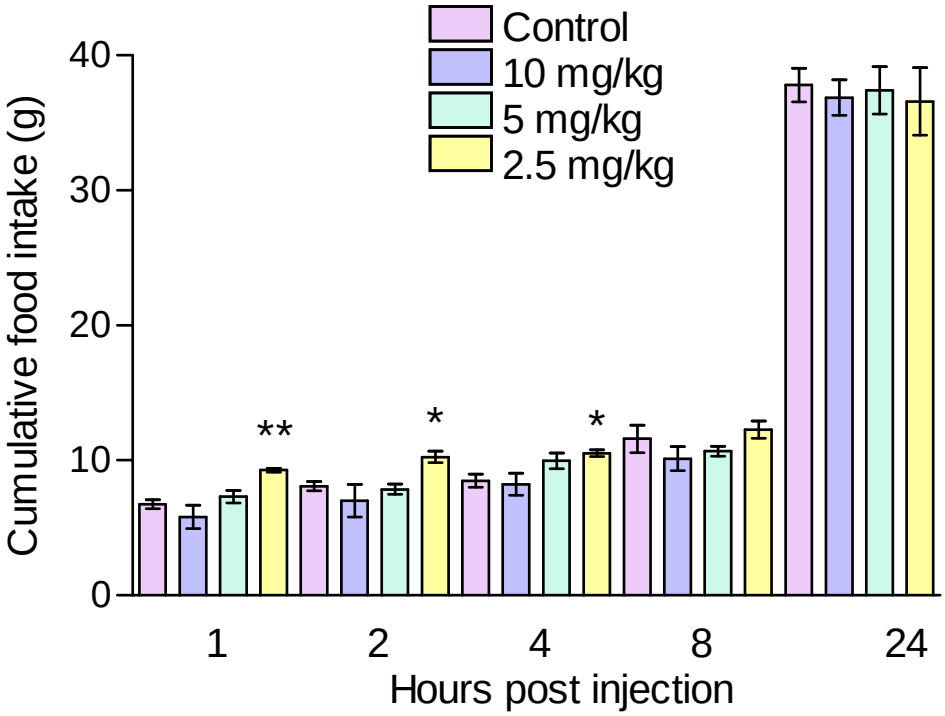

Figure 2.4.; Food consumption measured at 1, 2, 4, 8 and 24 hours after IP injection with imetit (2.5, 5 & 10 mg/kg, n = 6) or saline (n = 7) in 1 ml/kg. Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: ** $P < 0.005$, * $P < 0.05$.

It was observed that at one hour after treatment, rats that received imetit had eaten 8.77 ± 0.27 g of food, whereas those that received saline IP had eaten 6.75 ± 0.32 g ($P < 0.005$; figure 2.5). At 2 hours rats that received IP injections of imetit were still eating significantly more, with cumulative food intake at 9.77 ± 0.69 g compared with 8.09 ± 0.35 g for control rats ($P < 0.05$; figure 2.5). This significant difference in cumulative food intake continued up to 4 hours post injection, with the imetit group eating 10.28 ± 0.44 g and the saline group consuming 8.49 ± 0.48 g (figure 2.5). The statistical significance was lost between 4 and 8 hours post injection with rats receiving IP imetit injections having eaten 12.28 ± 0.64 g compared with 11.60 ± 1.01 g for the control group (figure 2.5).

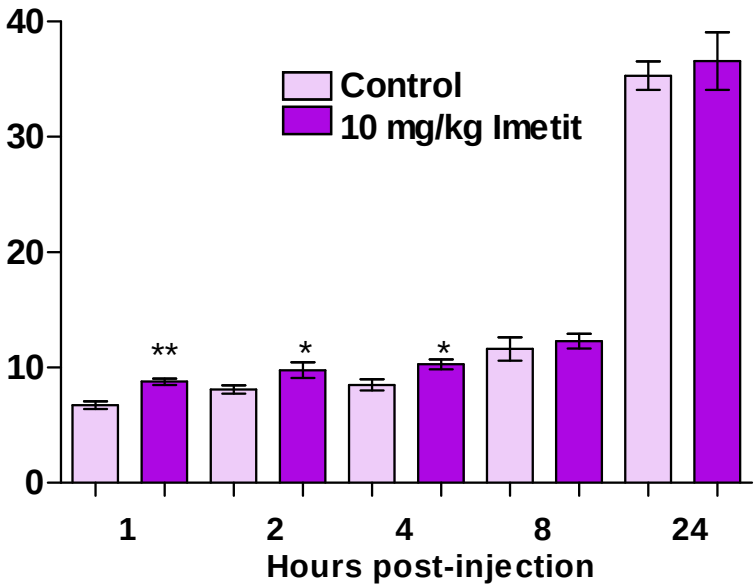

Figure 2.5.; Food consumption measured at 1, 2, 4, 8 and 24 hours after IP injection with 10 mg/kg imetit (n = 6) or saline (n = 7) in 1 ml/kg. Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: * *P < 0.05*.

2.4.3 H_3R inverse agonist effects on food intake in rats

After carrying out a dose response curve for thioperamide (an H_3R inverse agonist) given ICV at varying concentrations (400, 200, 100 nmol, figure 2.6), we found that a concentration of 400 nmol was the only concentration that we tested that gave a significant result (figures 2.6 and 2.7).

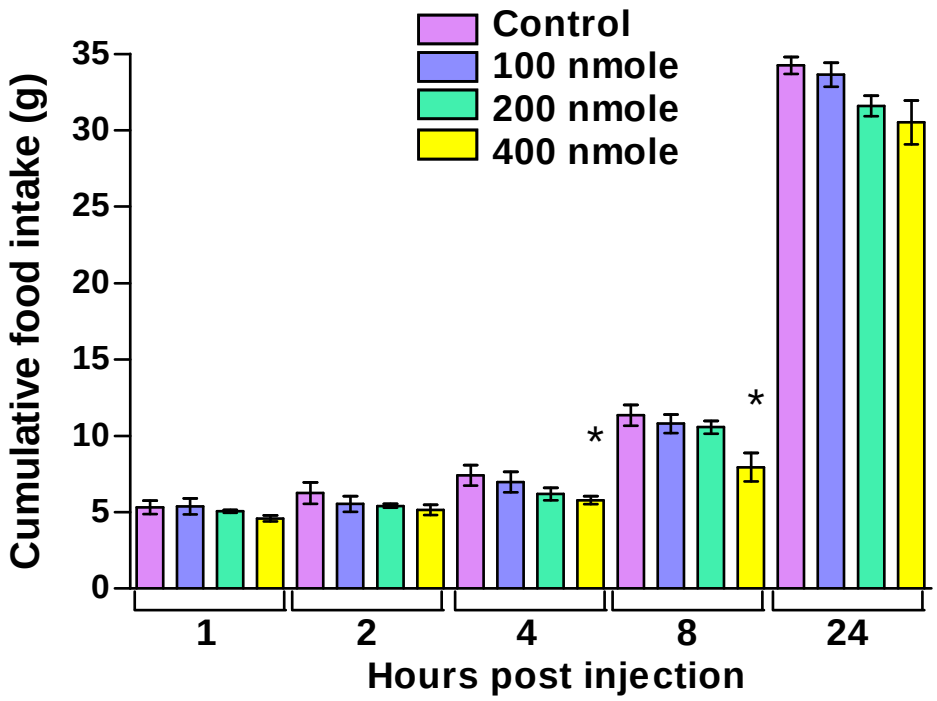

Figure 2.6.; Food consumption measured at 1, 2, 4, 8 and 24 hours after ICV injection with thioperamide (100, 200 & 400 nmol, n = 5) or saline (n = 5) in 2 µl. Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: * $P < 0.05$.

The ICV injection of 400 nmol thioperamide caused a significant decrease in cumulative food intake in rats compared with saline (figure 2.7), however, the difference was only significant at the 4- and 8-hour time points. At four hours, IP thioperamide-injected rats had eaten 5.80 ± 0.26 g and those that had received saline had eaten 7.43 ± 0.93 g ($P < 0.05$; figure 2.7). The significant difference in cumulative food intake between thioperamide and control rats was maintained at 8 hours, with control rats eating 11.35 ± 0.70 g whereas thioperamide-treated animals ate 7.79 ± 0.93 g. This significance was lost between 8 and 24 hours (figure 2.7). At 24 hours, the cumulative food intake of thioperamide-treated rats was 30.52 ± 1.43 g and for the saline-treated rats was 34.25 ± 0.56 g. These

feeding experiments suggest that the H_3R inverse agonist may have a delayed effect on feeding.

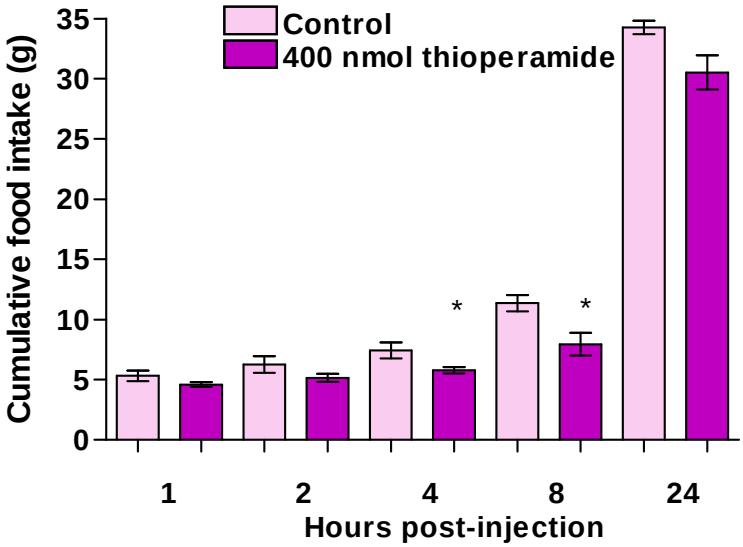

Figure 2.7.; Food consumption measured at 1, 2, 4, 8 and 24 hours after ICV injection with thioperamide (400 nmole, n = 5) or saline (n = 5). Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: * $P < 0.05$.

We then went on to determine the effect on food intake that thioperamide (H_3R inverse agonist) has when administered systemically at varying amounts by carrying out a dose response curve. The amounts investigated were, 2, 1, 0.5 mg/kg (figure 2.8). We found that giving thioperamide at all three amounts gave significant results at some time points post injection, with the highest concentration (2 mg/kg) giving the highest significance (figures 2.8 and 2.9).

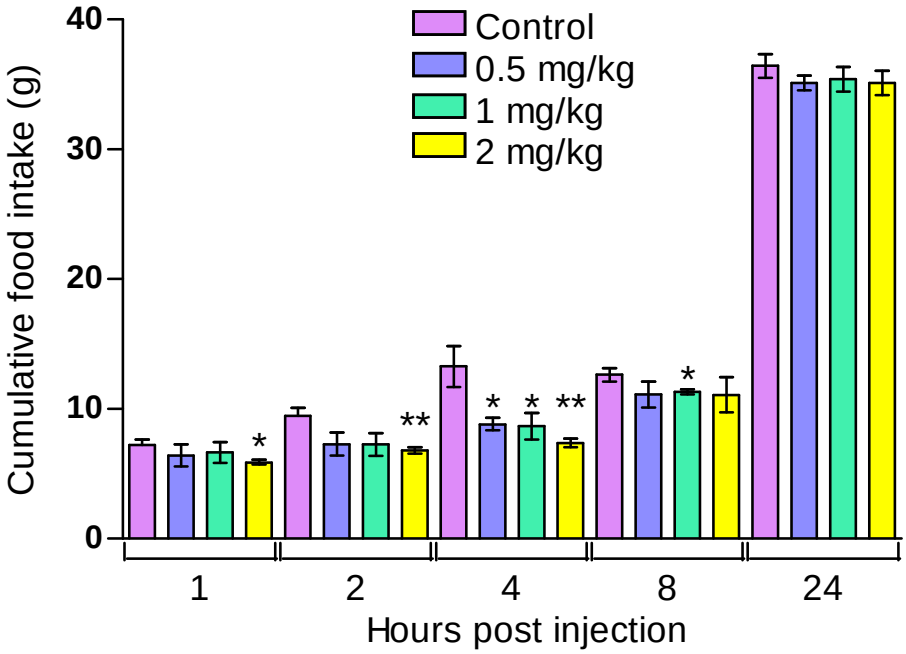

Figure 2.8.; Food consumption measured at 1, 2, 4, 8 and 24 hours after IP injection with thioperamide (0.5, 1 & 2 mg/kg, n = 5) or saline (n = 5) in ml/kg. Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: *** P < 0.005, * P < 0.05.*

The IP injections at 2 mg/kg caused a significant decrease in food intake in rats in the same way as the ICV study. One hour post injection, thioperamide-treated rats had eaten 5.89 ± 0.18 g and those treated with saline had eaten 7.22 ± 0.44 g ($P < 0.05$; figure 2.9). 2 hours post injection the significance of the difference increased to $P < 0.005$, and thioperamide-treated rats ate 6.80 ± 0.25 g compared with saline-treated rats that consumed 9.47 ± 0.63 g of food (figure 2.9). 4 hours post injection, rats that received a injection of thioperamide ate significantly less than rats receiving vehicle, with thioperamide-treated animals having eaten 7.39 ± 1.63 g and control animals consuming 13.22 ± 1.59 g of food (figure 2.9). The statisitical significance in difference in cumulative food intake was lost between 4 and 8 hours, with the H_3R inverse agonist-treated rats

consuming 11.09 ± 1.35 g and those that received saline eating 12.64 ± 0.51 g (figure 2.9).

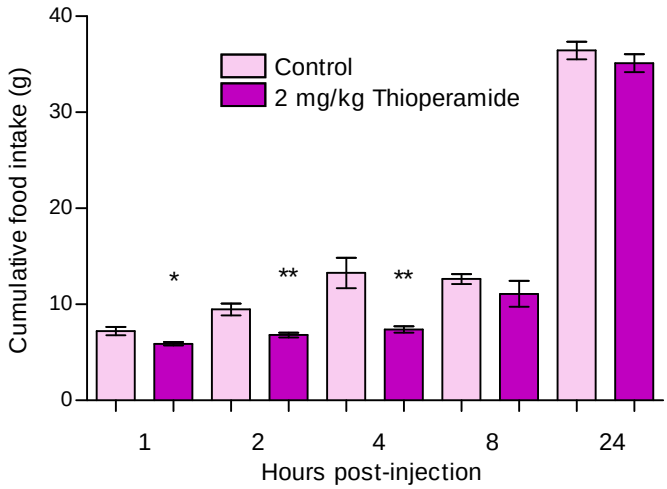

Figure 2.9.; Food consumption measured at 1, 2, 4, 8 and 24 hours after IP injection with thioperamide (2 mg/kg, n = 5) or saline (n = 5). Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: **** P < 0.0001*, *** P < 0.005, * P < 0.05.*

The i.p injections of thioperamide at 0.5 and 1 mg/kg also cause a significant reduction in food intake compared with control injections, but these doses appear to have a delayed affect on feeding, as significance was apparent only at 4-hours post injection (figure 2.8). This significance was lost for the 0.5 mg/kg dose by the 8-hour time point but rats that received a injection of 1 mg/kg thioperamide ate significantly less 8 hours post injection. Food intake normalised levels somewhere within the 8 and 24 hour time points. These feeding experiments correlate with the ICV thioperamide study showing the same effect on feeding.

A dose-response experiment for NNC1202 (an H_3R inverse agonist) given ICV at varying concentrations (100, 200 and 400 nmol, figure 2.10) was then carried out. We concluded that giving NNC1202 at all the concentrations tested showed no significant change in food intake (figure 2.10). There was no overall trend or change in food consumption suggesting that, when given ICV, NNC1202 has no effect on rodent appetite.

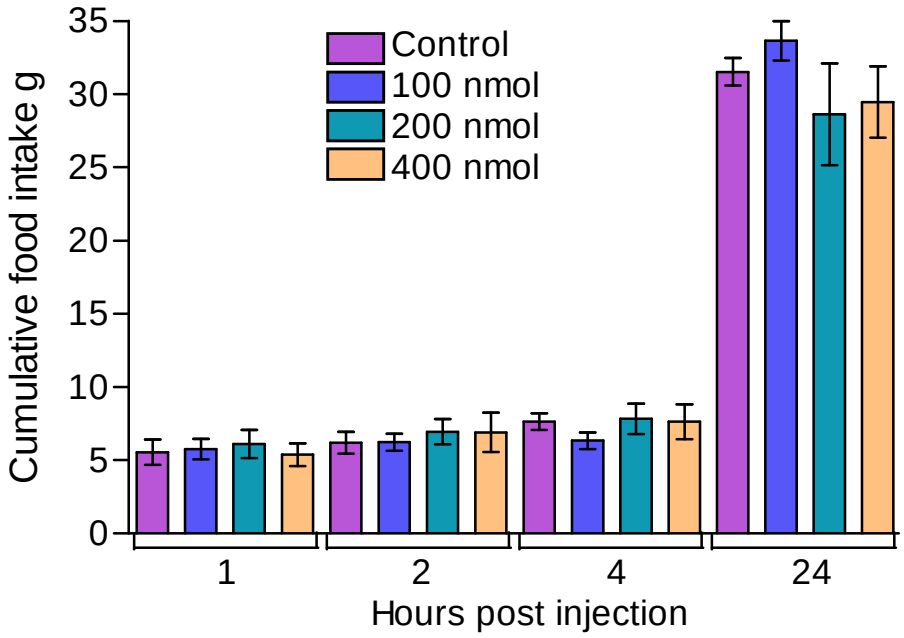

Figure 2.10.; Food consumption measured at 1, 2, 4 and 24 hours after ICV injection with NNC1202 (100, 200 & 400 nmol, n = 6) or saline (n = 8). Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: non significant.

We then carried out a dose-response curve for NNC1202 using IP injections (30, 20, 10 mg/kg, figure 2.11) to determine if the drug can affect food intake in rats when given systemically. We found that giving NNC1202 at all three doses showed a reduction in food intake, with the highest concentration (30 mg/kg)

showing the highest and most prolonged significance in food intake reduction (figures 2.11). The IP injections (30 mg/kg) caused a significant decrease in food intake in rats that lasted for a prolonged time and was still apparent 24 hours after the injection. One hour after injection, NNC1202-treated rats had eaten 1.03 ± 0.30 g and those that received saline IP had eaten 6.14 ± 0.55 g ($P < 0.0005$; figure 2.11). The significant difference between NNC1202-treated and saline-treated rats was still apparent after 24 hours, with rats injected IP with NNC1202 IPconsuming between 28.00 ± 0.91 g of food compared with saline treated rats that consumed 34.27 ± 0.93 g ($P < 0.0005$; figure 2.11). These results suggest that the H_3R inverse agonist NNC1202 given IP at 30 mg/kg and 20 mg/kg has a dramatic affect on the food consumption of rats causing a much reduced food intake that is prolonged and still apparent after 24 hours post injection.

We noted that NNC1202 given at 20 mg/kg also caused a significant reduction in food intake at 1, 2, 4 and 24 hours post injection. Also our results also indicate that when NNC1202 is administered at 10 mg/kg a significant reduction is apparent after 1 and 2 hours post injection. These data suggests that even at the lower doses NNC1202 has a significant effect on food consumption in rats.

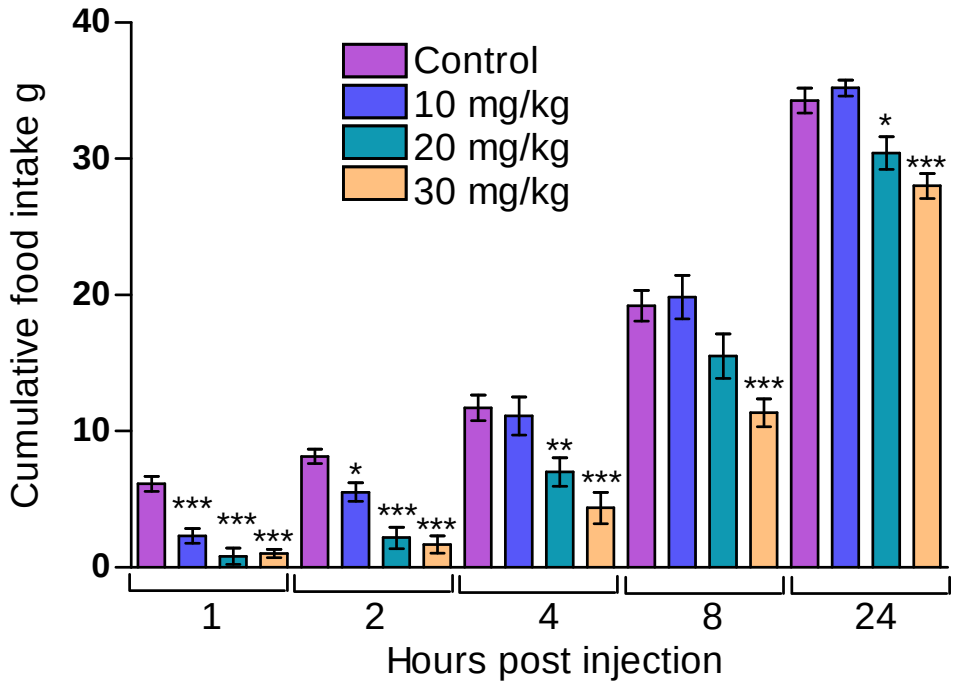

Figure 2.11.; Food consumption measured at 1, 2, 4, 8 and 24 hours after IP injection with NNC1202 (10, 20 & 30 mg/kg, n = 7) or saline (n = 7). Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: *** $P < 0.0005$, ** $P < 0.005$, * $P < 0.05$.

2.4.4 The effect of the H_3R compounds on the behavioural satiety sequence

Similar to the earlier experiments described in this chapter, animals ate significantly more food when given the H_3R agonist imetit (figure 2.12) compared with all other groups. When given either of the H_3R inverse agonist drugs, thioperamide or NNC1202, rats significantly reduced their food intake (figure 2.12). Measurements of food intake were made at the end of the 90-minute observation period. As mentioned, food intake was measured 90 minutes into the lights off period showing that the changes in food intake seen after administering these drugs occurs both in pre-fed and fasted animals in both lights on and lights off periods.

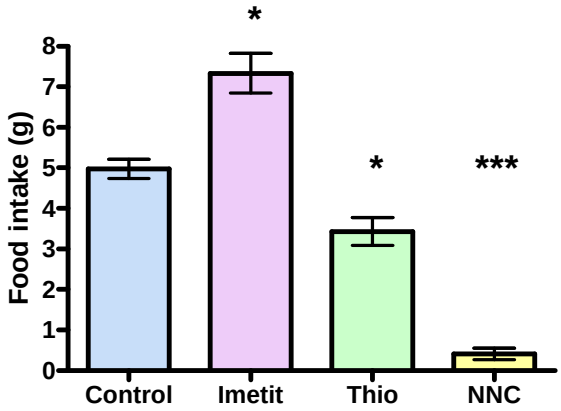

Figure 2.12.; Food consumption measured at 90 minutes after IP injection with imetit (10 mg/kg), thioperamide (2 mg/kg), or NNC1202 (30 mg/kg) or saline (n = 8 for all groups). Data are expressed as mean ± S.E.M. One-way ANOVA with Bonferronni *post hoc* test was carried out: * $P < 0.05$, *** $P < 0.0005$.

Figure 2.13 shows the BSS control group animals display a normal sequence of behaviour. They initially showed episodes of feeding interspersed with episodes of activity, before progressing to extended periods of grooming and rest, as they became satiated. Apart from an initial increase in eating behaviour in the first few bin points, the imetit-treated animals showed a similar pattern to the control animals. Also, the pattern of behaviours was similar in the thioperamide-treated animals apart from slightly less time spent eating over the first few time bins. These results show that no unusual behaviours, e.g. immobility, malaise or sickness behaviour were noted in these treated groups. This was not the case for the NNC1202-treated group which showed a very different pattern of behaviours. Rats treated with NNC1202 showed prolonged episodes of inactivity where they were observed as quiet, motionless and unresponsive, although with eyes open, leading to assumption they are suffering from malaise. They appeared to recover slightly towards the latter part of the 90 minutes post injection and were even seen to start feeding in the last few bin time points. These results suggest that the

drug is having an effect on the animals that is making them feel unwell or unmotivated and as a result of this they are not eating as much as the other groups.

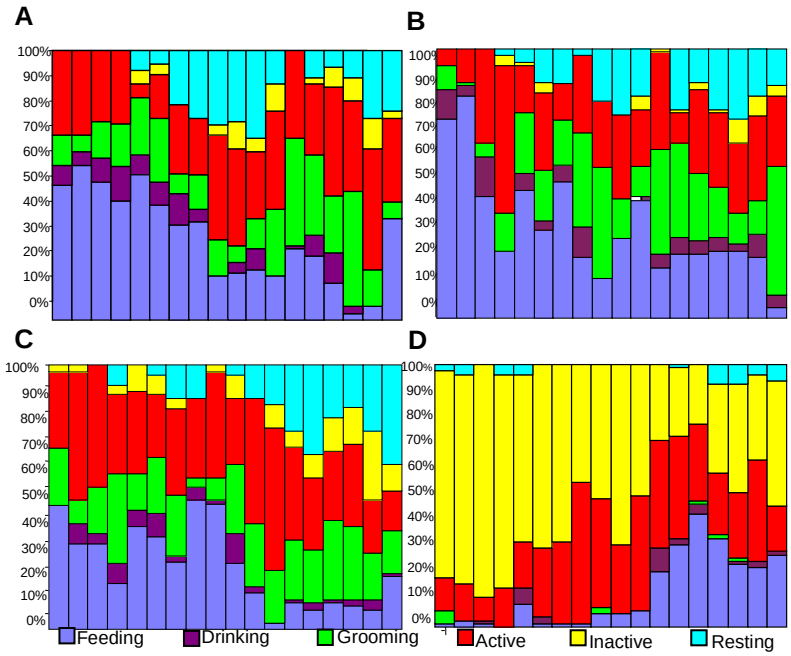

Figure 2.13.; Behavioural satiety sequence – animals were IP injected with, A. saline, B. imetit, C. thioperamide, or D. NNC1202 and introduced to food 90 seconds post-injection. Their behaviours were scored every 30 seconds for 90 minutes according to the categories mentioned in the method section. Data are collated into 5 min time bins and expressed as mean percentage of total behaviour.

	Saline	Imetit	Thioperamide	NNC1202
Feeding	31%	34%	24%	13%
Drinking	6%	5%	4%	2%
Grooming	18%	21%	20%	1%
Active	29%	26%	33%	28%
Inactive	4%	2%	7%	54%
Resting	13%	12%	12%	2%

Table 2.2.; The overall average percentage of time spent in each behaviour over the 90-minute observational period whilst carrying out the behavioural satiety sequence after animals were injected with either, saline, imetit, thioperamide, or NNC1202.

Animals that received an injection of imetit at lights out spent a slightly longer percentage of their time eating compared with control animals over the 90 minutes observation period. Imetit treated animals spent 34 ± 3% of their time eating, whereas control animals spent 31 ± 3% (table 2.2). All time spent carrying out other behaviours corresponded with control animals. Thioperamide-treated subjects also showed similar behaviours as controls and spent a similar percentage of time in each behaviour. The one behaviour to deviate from control levels was the time spent feeding. Thioperamide-treated animals spent 24 ± 3% of their time feeding over the 90 minutes they were observed. Conversely, NNC1202-treated subjects spent a large percentage of their time in different behaviours during each time bin compared with the control animals and the thioperamide- and imetit-treated animals. Firstly, the NNC1202 group showed a much lower percentage of time feeding compared with controls or thioperamide-treated subjects, with only 13 ± 2% of their time being spent feeding. Also,

animals that received an injection of NNC1202 spent the majority of their time during the 90 minute observation period inactive with signs of malaise (54 ± 4%). When compared with control animals, which spent only 4 ± 1% of the 90 minutes showing inactivity, it is apparent that the NNC1202 animals are clearly showing a very different pattern of behaviours, as they are when compared with animals that received either the imetit or thioperamide injections.

A measure of the induction of satiety may be inferred from the time during the observational period when resting overtakes feeding as the dominant behaviour. The control saline-, imetit- and thioperamine-treated animals all showed similar times taken for rest to become the dominant behaviour. The point of transition or 'cross over' took place in time bins 8 to 11 for animals on the three groups (figure 2.15). The NNC1202 treated animals showed a very different BSS compared to all other groups. It was noted that these animals were highly inactive for prolonged periods of time following injection and only started feeding towards the end of the 90 minutes. There was no real transition from feeding to resting with the animals spending very little time resting or feeding.

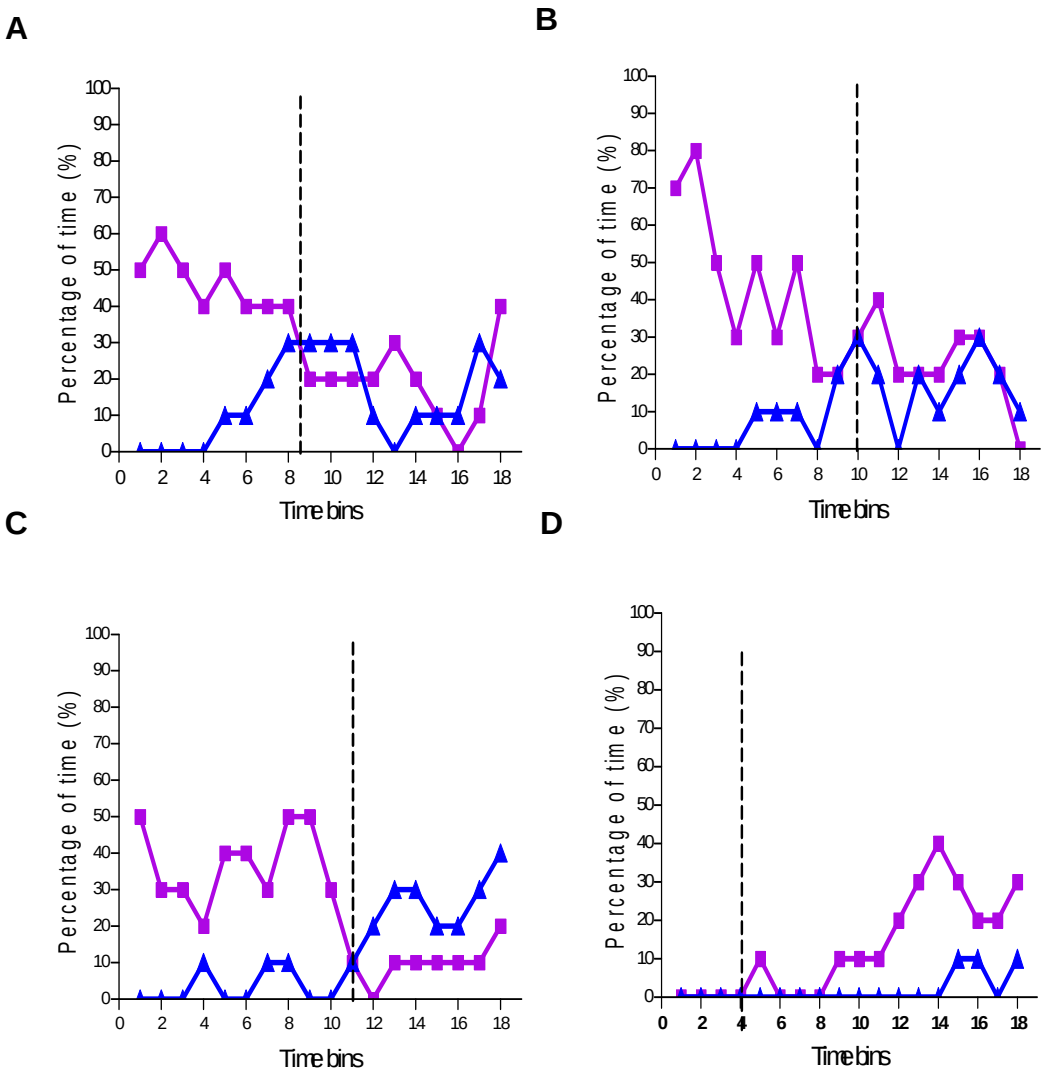

Figure 2.15.; This figure illustrates the observational period when resting overtakes feeding as the dominant behaviour after animals were injected with, A. saline, B. imetit, C. thioperamide, or D. NNC1202. Where the two lines first overlap is known as the 'cross over' point. The purple line indicates feeding behaviour and the blue line indicates resting. Each time bin represents 5 minutes.

2.4.5 Do imetit and thioperamide have similar effects on feeding in mice?

Using similar concentrations to those used by Yoshimoto *et al.* (2006), we administered two doses of imetit to determine whether this H_3R agonist had the same effects on food intake that we and others have reported in rats or whether it had the opposite effects noted by Yoshimoto *et al.* (2006). We gave 10 or 20 mg/kg of imetit IP to mice, 10 mg/kg had no effect on food intake either at 1 hour or 2 hours post injection. 20 mg/kg did have a significant effect on food intake resulting in significantly less food being eaten by imetit-treated mice compared with the saline-treated animals (figure 2.16). This significance was only apparent in the first hour post the imetit injection with the imetit treated group only eating 0.41 ± 0.05 g compared to control animals that ate 0.74 ± 0.11 ($P < 0.01$) (figure 2.16). This significance was lost by two hours post injection, with control animals eating 1.14 ± 0.11 g and imetit treated mice eating 0.08 ± 0.11 g (figure 2.16). Thus, we found in contrast to what we noted in rats, imetit reduce food intake in mice.

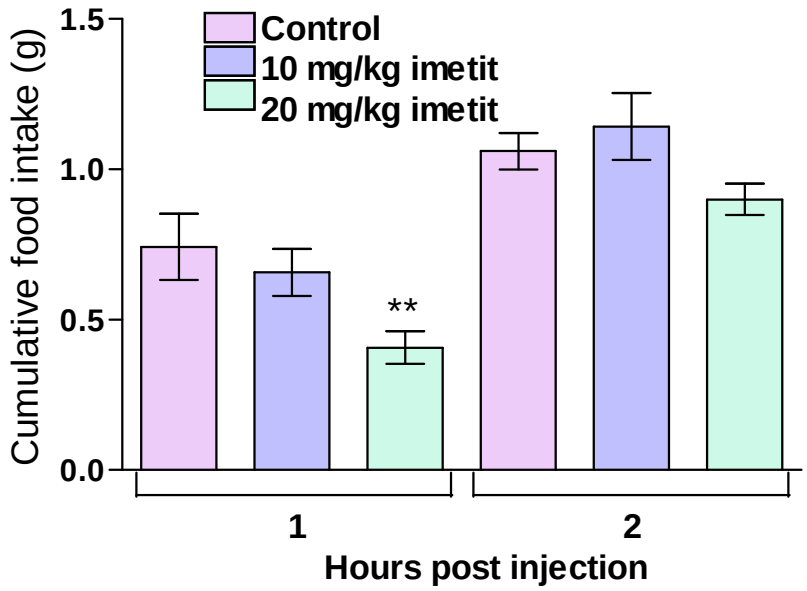

Figure 2.16.; Food consumption measured at 1 & 2 hours after IP injection with 10 & 20 mg/kg imetit or saline (n = 5). Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: ** $P < 0.01$.

We then wanted to determine whether the H_3R inverse agonist thioperamide had opposing effects on food intake in mice and rats. We administered both 10 and 20 mg/kg of thioperamide, and measured food intake for two hours after the injection (figure 2.17). We found both doses resulted in a significant reduction in food intake. 10 mg/kg of thioperamide resulted in anorexia in mice for the first hour post injection. Here we found that control animals ate 0.74 ± 0.11 g of food, whereas thioperamide-treated animals ate 0.51 ± 0.06 g of food ($P < 0.01$). This significance was lost between 1 and 2 hours post injection with mice administered, with imetit mice ate 1.14 ± 0.11 g of food whereas the control grouped ate 0.90 ± 0.09 g of food 2 hours post injection (figure 2.17). Mice given a dose of 20 mg/kg of thioperamide showed significant hypophagia at both the 1 and 2 hour time points. Mice given 20 mg/kg ate 0.027 ± 0.06 g of food 1 hour post injection, which is significantly less food ($P < 0.001$) than animals that received a injection of saline, which ate 0.74 ± 0.11 g. This significance was still apparent at two hours post injection, with the control group having eaten 1.14 ± 0.11 g of food compared with thioperamide-treated animals that ate 0.68 ± 0.10 g (figure 2.17). Thus, we found thioperamide to be a powerful and effective anorexic inducing drug in mice.

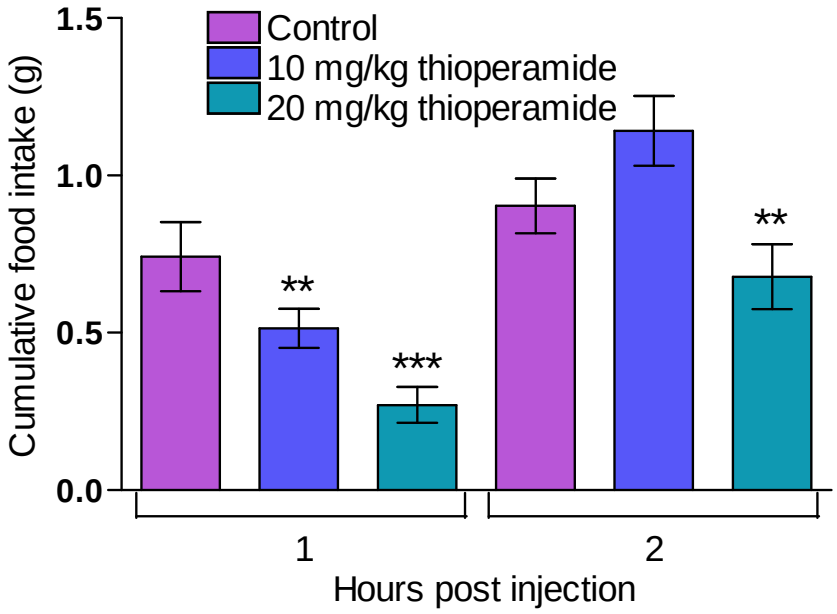

Figure 2.17.; Food consumption measured at 1 & 2 hours after IP injection with 10 & 20 mg/kg thioperamide or saline (n = 13). Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferonni *post hoc* test was carried out: *** $P < 0.001$, ** $P < 0.01$.

2.5 Discussion

2.5.1 Histamine causes a reduction in feeding in rats

In the first experiments described, histamine was injected into the lateral ventricle of the rat brain, close to the hypothalamus, an area associated with appetite. It has been shown previously by a number of research groups that histamine can decrease food intake in rodent models following ICV, IP and intravenous (IV) injections (Doi *et al.* 1994; Endou *et al.* 2001; Lecklin *et al.* 1998), and we aimed to confirm these studies by determining if we could also cause a decrease in food intake in male Sprague-Dawley rats. We found that ICV histamine injection caused a significant decrease in food intake for up to 8 hours post injection. 200 nmol histamine caused a reduction in the food eaten by rats

compared with rats receiving a saline vehicle injection. Intake was normalised by 24 hours post injection. That is, histamine-injected rats compensated with an increase in consumption at later time points, indicating that the histamine did not have long-term deleterious effects.

According to the literature, H_1R and H_3R can mediate effects on feeding behaviour in rodents, with H_1R agonism causing a reduction, and H_3R agonism causing an increase in food intake, respectively. As our ICV histamine injections cause a reduction in food intake, the simplest explanation is that histamine is acting directly on postsynaptic H_1R, perhaps within the hypothalamus. H_1R are highly concentrated in the hypothalamus (Ookuma *et al.,* 1993; Doi *et al.,* 1994), and direct injections of histamine or H_1R agonists into either the VMN or PVN can reduce feeding (Kinnunen *et al.* 1998).

However, it remains possible that histamine's actions could be indirect and not the result of activating hypothalamic H_1Rs. H_3R occur densely within the hypothalamus and can act as heteroreceptors. Thus, giving injections of histamine could activate H_3R heteroreceptors present on neurones containing other transmitters to affect the release of these transmitters, which could indirectly affect the food consumption of the animals being tested (Schlicker *et al.,* 1993, Yoshimatsu et al., 2008).

2.5.2 H_3R agonism effects on food intake in rats and mice

Our findings, on the effects of the H_3R agonist imetit on food intake in rats is in agreement with the vast majority of the literature already published. Many studies have been carried out that suggest H_3R agonists, including imetit, cause an increase in food consumption (Garbarg *et al.,* 1992; Leurs *et al*., 2005; Hancock *et al*., 2004). We too found that the H_3R agonist imetit, when administered either centrally or systemically caused a significant increase in food intake in male

Sprague-Dawley rats. The majority of H_3R are expressed centrally (Lovenberg *et al.*, 1999**)** and, therefore, these results support the assumption that imetit can cross the blood-brain barrier to affect central H_3R receptors to affect feeding (Lecklin *et al.,* 1998). We could assume that administering a H_3R agonist would increase the auto-regulatory nature of H_3R autoreceptors and, thus, endogenous circulating histamine would be reduced resulting in an increase in food intake.

However, one study has found imetit to have opposite effect on food intake in mice (Yoshimoto *et al.,* 2006). This study suggests imetit causes a decrease rather than an increase in food intake in mice and that continual administration can result in body weight reduction. Thus, our next aim was to carry out a feeding study in mice to determine whether this research groups finding are in disagreement with the rest of the literature, or whether species related difference in the effects of this H_3R drug actually causes it to have an alternative effect in mice. Our results agreed with the findings of Yoshimoto and colleagues with imetit causing a significant reduction in food intake in mice when given systemically. These data supports the controversial findings of Yoshimoto *et al.* (2006) and suggest imetit can have opposing effects in rats and mice and again illustrates the possible differences in effects H3R drugs can have amongst different species.

Histamine is known to play a crucial role in sleep-wake control and it has been reported (Parmentier *et al.,* 2002; Lamberty *et al.,* 2003) that several H_3R agonists, including imetit, increase slow-wave sleep by inhibiting histamine release. Thus, it has been suggested that H_3R agonists might have hypnotic actions, and could possibly explain the hypophagic effects of imetit reported (Parmentier *et al.,* 2002). However, Yoshimoto *et al.* found that imetit did not significantly reduce locomotor activity compared with vehicle (Yoshimoto *et al.,* 2006). Likewise, the hyperphagic effects seen in rats given imetit and our BSS findings, which showed no significant alterations in any behaviour other than

feeding, suggests movement and/or wakefulness was not seriously affected. It would be important to carry out the BSS study in mice too.

2.5.3 H_3R inverse agonism decreased food intake in rats and mice

Although some research groups have found thioperamide to have varying effects (Itoh *et al.,* 1998; Sindelar *et al*., 2004), it is a widely investigated H_3R inverse agonist and has been shown to decrease food intake by a number of research groups (Lecklin *et al*., 1998; Sakata *et al.,* 1990). NNC1202, on the other hand, is a more recently developed H_3R inverse agonist and has been much less widely investigated, yet it is reported to have robust inhibitory effects on food intake (Wulff *et al.,* 2002).

We carried out feeding studies to determine how these H_3R inverse agonists would affect food intake in male Sprague-Dawley rats when administered either centrally or sytemically. By both routes of administration, thioperamide caused a significant decrease in food intake in rats. By 24 hours post injection of thioperamide by either route, cumulative food intake was no longer significantly different compared with vehicle controls, suggesting that the drug is not having a long-lasting effect on food intake and no major adverse actions. This is backed up by our BSS results which showed that the sequence of post feeding behaviours did not greatly differ from those seen in control animals, with the only difference in behaviour being seen in the feeding behaviour. Although we found the behaviours of the rats that received thioperamide did not greatly deviate from those given saline there was no shift in the sequence, which might suggest thioperamide is not affecting satiation directly.

The H_3R inverse agonist, NNC1202, a compound donated by our industrial partners, Novo Nordisk, has been shown to decrease food intake in rodent and primate studies when given systemically (Wulff *et al.,* 2002). We were able to

confirm this. However, to date there is no evidence that shows the compound to have the same effects when given by the ICV route, which would narrow its effectiveness to a central site of action. We found NNC1202 to have no observable effect on food intake at any of three doses tested (100, 200 or 400 nmol) when injected ICV. No data are available to indicate the efficacy of NNC1202 compound when given ICV, and it is possible that insufficiently high doses were chosen. Since the hypothesis set out by the company is that the compound is crossing the blood-brain barrier to cause anorexia when administered peripherally, it is surprising that NNC1202 is ineffective at any of our tested doses. However, NNC1202 did cause a dramatic reduction in food intake when injected IP and cumulative food intake was still significantly reduced at 24 hours post injection. This suggests this compound has longer-term effects on feeding. We did not measure food intake after the 24 hour period and thus can not define when, if at all, cumulative food intake normalised. However, we did note that the rats did not lose weight and showed comparable weight gain to control rats over the next seven days. However, a question remains whether it is achieving a reduction in appetite through the hypothesized mode of action (i.e. the central release of endogenous histamine) or whether it is creating an adverse reaction within the periphery that leads indirectly to hypophagia. Our BSS experiments show NNC1202 treated animals eat very little and are inactive for the majority of the 90 minutes they were observed, suggesting NNC1202 is indirectly affecting appetite and may be affecting other behaviours causing a lack of food intake. Unfortunately, our BSS studies do not distinguish between possible causes for the recorded inactivity (e.g. malaise, sedation etc). An alternative study to carry out would be a conditioned taste aversion test to investigate the development of aversion and, thus, malaise or nausea induction after the administration of a drug. Suffice to say that the enormous disruption of the BSS and the lack of effect when given centrally but dramatic affect when peripherally

administered, suggests that NNC1202 is having undesirable adverse actions and, thus, we decided not to continue with our studies using this drug. In fact, Novo Nordisk have since stopped its development programme on this and other small molecule drugs in this field.

It has certainly been shown that a number of H_3R inverse agonists have opposing effects on food intake in different animal models (Lecklin *et al.,* 1998; Hancock *et al.,* 2004; Jethwa *et al.,* 2009). Hancock *et al.* Showed that the H_3R antagonist, A-133144, decreased weight and body fat when given orally over a 28-day period, although surprisingly minimal food intake consumption changes were observed (Hancock *et al.,* 2004). Lecklin *et al.* (1998) found that giving thioperamide centrally had no affect on food intake, whereas Jethwa and colleagues showed that in the seasonal hamster administering H_3R inverse agonists in the lean state reduced the animals food intake (Jethwa *et al.,* 2009). Alternatively Yoshimoto *et al.* found that thioperamide given to mice resulted in significant weight gain and hyperphagia (Yoshimoto *et al.,* 2006).

After determining that the H_3R agonist imetit can cause an increase in food intake in rats and yet produces anorexia in mice we wanted to determine whether thioperamide could also have these differing effects in different animal species as shown by Yoshimoto *et al.* (2006). We found, when given systemically, the H_3R inverse agonist thioperamide caused a significant decrease in food intake in mice at both 10 and 20 mg/kg. These findings support our data from our rodent studies which also suggest thioperamide acts as a potent hypohagic agent, but oppose the results previously published by Yoshimoto and colleagues. The difference in our findings and the findings of Yoshimoto *et al.* (2006) are not entirely surprising as thioperamide has long been controversial in its effectiveness with varying effects seen by a number of groups being published. Some suggest it causes anorexia (Arrang *et al.,* 1987), whilst others suggest it has no effects on food intake (Itoh *et al.,* 1998), and others suggesting its effects are determined by the

route of administration (Sindelar *et al*., 2004). We used CD-1 mice whereas Yoshimoto and colleagues carried out their experiments on C57BL/6J mice. The differences in mouse strains could explain the differences in our findings especially as a number of other H_3R inverse agonists have shown different effects in their actions depending on the species being tested (Oishi *et al.,* 1983; Hancock *et al.,* 2004; Tokita *et al.,* 2006).

Our results from systemic injections suggest that imetit and thioperamide could be crossing the blood-brain barrier as they have strong effects on food intake. This is useful for the development of pharmaceutical agents as it would be impossible to administer drugs centrally to human patients. Furthermore, since H_3R are found in abundance centrally, the release of endogenous histamine or other transmitter modulation within the brain is unlikely to have “off-target” effects in the periphery. It is interesting that we found no evidence for adverse actions of either drug from our BSS. Altogether our evidence suggest, although more research needs to be carried out, H_3R might still be worthy of consideration as a useful target for the treatment of obesity or other eating disorders.

2.6 Future work and directions

The H_3R can act as an autoreceptor to modulate histamine synthesis and release or as a heteroreceptors on the terminals of other transmitter-containing neurones (Schlicker *et al.,* 1994). From the studies carried out we are not able to define whether the increase in food intake seen in rats with imetit or the decrease in feeding seen with thioperamide was due to effects on the presynatic H_3R autoreceptor or the H_3R heteroreceptor. This could be determined by carrying out feeding studies where the H_3R based drugs are given along with antagonists for

post-synaptic histamine receptors or for other transmitters. The situation is further complicated by the fact that histamine can also co-localise with other transmitters that have effects on feeding and appetite, such as dopamine (Fulton S., 2010, Campbell *et al.,* 2007), GABA (Gruninger *et al.,* 2007, Patel *et al.,* 2010) and serotonin (Ebenezer *et al.,* 2007, Halford *et al.,* 2010).

To determine that diurnal influences are not having effects on drug administration and determining different behaviours, we also could examine the behavioural satiety sequence following histamine injection at different times of the day. Any disruption of the sequence would indicate unusual actions of a treatment that might be affecting food intake indirectly, for example of aversion, nausea or sedation. Future work might also include the use metabolic cages to determine how histamine can affect energy metabolism.

These feeding studies allowed us to determine how the drugs affected food intake in adult rats but didn't give us any information on where the drugs were acting. Thus, our next step was to carry out immunohistochemistry studies to measure the induction of c-*fos*, a neuronal marker for activation, to determine exactly where in the adult rat brain the H_3R drugs are acting.

Chapter 3:

Neuronal activation by histaminergic receptor ligands

3.1 Introduction

3.1.1 Histamine and its neuronal projections throughout the rat brain

Histamine is a central neurotransmitter and, as mentioned previously, is involved in many behavioural and physiological functions. The histamine neurones are found solely in the TM of the posterior hypothalamus (Schwartz *et al.,* 1991), from here the histaminergic nerve fibres project to almost the entire areas of the brain through dorsal and ventral pathways, with the highest density of projections being found within the hypothalamus, in particular to the VMN. Although all brain regions receive some histaminergic innervations, some receive a much denser input than others. An over-simplified description would be to say that that structures innervated by the ventral ascending pathway receive a powerful input whereas those innervated by the dorsal ascending pathway tend to have a much lower density of fibres (Brown *et al.,* 2001). The cerebral cortex of the rat brain has a moderate density of fibres, whilst the histaminergic innervation of the thalamus is concentrated upon the periventricular nuclei. Although the hippocampus receives only a moderate innervation by histamine neurones, histamine has strong effects on excitability in this area (Haas *et al.,* 1983; Haas *et al.,* 1986; Panula *et al.,* 1989; Greene *et al.,* 1990). Also a low to moderate level of innervation is present in the striatum and nucleus accumbens (Brown *et al.,* 2001). Projections to the midbrain, brain stem, cerebellum and spinal cord are lower concentration than the ascending projections. All of the other aminergic cell groups receive at least a moderate density of fibres, with the substantia nigra and ventral tegmental area being strongly innervated. In addition, prominent projections have been noted in the inferior and superior colliculi, periaqueductal gray, nucleus of the trigeminal nerve and nucleus of the tractus solitarius (Brown *et al.,* 2001).

Histaminergic axons do not in general form synaptic specialisations, instead histamine is released from varicosities (swellings containing synaptic vesicles) located periodically along the axon (Takagi *et al.,* 1986). Thus, histamine release sites and histamine receptors are not always directly apposed to one another. Therefore, histamine can function like a local hormone, acting on neurones, glial cells and blood vessels in a concerted manner (Wada *et al.,* 1991, Brown *et al.,* 2001). Although some brain regions receive only a low density of histaminergic fibres there is a considerable inconsistency between histaminergic fibre density and histaminergic receptor density in various brain regions (Schwartz *et al.,* 1991) and the prominence of electrophysiological effects (Takagi *et al.,* 1986). This suggests that all brain regions can be affected to a lesser or greater degree depending on the situation. For example, areas with a high histaminergic density of fibres will probably be affected tonically during waking, whilst areas with lower histamine fibre density may only be affected under certain behavioural situations when histamine neurones fire more rapidly.

There is accumulating evidence that histaminergic neural circuits arising in the TM and projecting into the satiety centres of the hypothalamus participate in regulation of energy homeostasis. The central function of neural histamine in regulation of food intake is further underlined by the fact that leptin, amylin and bombesin have been suggested to exert at least some of their anorectic effects through the histaminergic circuitry (Malmlof *et al.,* 2005). Also, histaminergic neurones project into hypothalamic centres known to be involved in food intake regulation, these include the PVN and VMN, where the anorectic effect is thought to be mediated by the postsynaptic histamine H_1R. Malmlof *et al.* suggest the density of the H_1R, together with the H_3R-mediated control of the intrasynaptic concentration of histamine, are both crucial in determining the strength of the anorectic signal (Malmlof *et al.,* 2005).

Although there are only two histamine receptors (H_1R and H_3R) known to influence feeding behaviour there are a number of other histamine receptors that, like H_1R and H_3R, are found widely both peripherally and centrally. The ubiquitous nature of histamine receptors is one of the main reasons that histamine has such diverse and widespread effects. For this reason we began by giving histamine i.c.v. to determine the exact effects it has acting purely upon the brain. This stops any peripheral receptors being stimulated and potentially causing secondary actions in the brain through afferent projections. We then went on to determine the effects of the histamine agonist and histamine antagonists by carrying out both i.c.v. and i.p. injections. We carried out i.p. injections as wanted to see the effects the drugs would have if given systemically and we needed to determine if the drug was able to cross the blood-brain barrier. This is important in considering the possible route of administration in a clinical setting.

3.1.2 The neuronal activation marker c-fos

Immediate-early genes (IEG) show rapid and transient expression, are activated upon cell stimulation, and their expression cannot be prevented by protein synthesis inhibitors (Sheng *et al.,* 1990). IEG's are believed to encode transcription factors which modify the expression of other target genes (Sheng *et al.,* 1990). The induction of these genes by neuronal activity can result in long-lasting changes and even modify the phenotype of the cell. One of the more commonly studied and understood IEG is c-*fos* (Herrera *et al.* 1996). A number of studies have demonstrated that increased neuronal activity *in vivo* induces c-*fos* expression (Morgan *et al.,* 1987, Hunt *et al.* 1987, Ons *et al.,* 2010), leading to c-*fos* expression commonly being used to determine which neuronal populations are activated following different stimuli (Dragunow *et al.,* 1989), such as drug administration.

C-*fos* is the most widely used functional anatomical marker of activated neurones within the CNS, mainly because it is expressed at relatively low levels under basal conditions; it is induced in a stereotypical manner in response to extracellular signals; the response is transient; detection of c-*fos* expression is relatively basic and it can be easily combined with a number of other markers (Elias et al., 1999; Zhang et al., 2000). C-*fos* and its protein product c-Fos are generally thought to act as reliable markers for identifying activated cells (Kovas KJ., 2008), but it must be noted that the lack of a Fos response does not rule out the involvement of the cell group or tissue area being investigated (Ericsson *et al.,* 1994). Although, if depolarisation alone regulated c-fos expression, its detection would be observed in neurones throughout the brain, even under basal conditions. Thus, it is likely that only changes in afferent inputs and changes in external stimuli induce c-*fos* expression (Scott *et al.,* 2007; Kovas *et al.,* 2008; Dodd *et al.,* 2009).

In most cell types, c-Fos protein levels are relatively low under basal conditions (Curran *et al.,* 1988). However, stimuli linked to cell division, such as serum and polypeptides can stimulate c-*fos* expression (Curran *et al.,* 1985; Greenberg *et al.,* 1986; Greenberg *et al.,* 1984; Sheng *et al.,* 1990). Other signals associated with neuronal excitation can also elicit, albeit temporary, c-Fos expression. For example, voltage-dependent Ca^{2+} channel activation and neurotransmitter release (Greenberg *et al.,* 1986). Activation of c-*fos* in the brain can be induced by a diverse group of stimuli and therefore c-*fos* has been used as a tool to study neuronal activation in a number of different systems in the brain (Herrera *et al.,* 1996). Hence, C-*fos* is an important tool in science and its detection has led to a further understanding of different systems in the CNS and allows the mapping of neuronal populations activated by a variety of external stimuli.

3.2 Objectives

3.2.1 Where does i.c.v. injection of histamine activate cells within the rat brain?

A number of brain areas are known to be central in the control of appetite and feeding behaviour. Thus, using c-Fos immunohistochemistry, we aimed to determine which brain areas were activated when histamine was administered centrally and to establish how widespread the activation was.

3.2.2 Do i.p. injections of a histamine agonist and inverse agonist cause activation of cells in similar areas to that of histamine?

Due to the significant changes on feeding behaviour caused by the administration of the H_3R agonist, imetit, and the H_3R inverse agonists, thioperamide and NNC1202, we also wanted to determine if the same brain areas were activated when these drugs were given systemically.

3.3 Methods

3.3.1 Animals

Male Sprague-Dawley rats (250-280 g; Charles-River, UK) were adapted to a 12 h/ 12 h light-dark cycle (lights on 08.00-20.00) a week before surgery or before their intraperitoneal (i.p.) injections. These and all other experiments conformed with the Home Office Scientific (Animal) Procedures Act of 1986 and local ethical review.

3.3.2 Compounds

Histamine (200 nmol) was administered via injection into the lateral cerebral ventricle (intracerebroventricular administration, i.c.v.), thioperamide (2 mg/kg body weight), imetit (10 mg/kg) and NCC-0038-1202 (NNC1202, 30 mg/kg) were administered intraperitoneally. All compounds were diluted in isotonic saline to the required concentration. Histamine, thioperamide and imetit were purchased from Sigma-Aldrich Company Ltd (Dorset) and NNC1202 was donated by Novo Nordisk.

3.3.3 Experimental procedures for i.c.v. cannulation

Rats were anaesthetised with 2 % isofluorane in O_2 at a rate of 1 l/min. Once their heads had been shaved, rats were placed securely in the stereotaxic apparatus (Stoelting, Illinois, USA). A 2-3 cm incision was made along the midline of the cranium exposing the skull. All connective tissue was displaced from the cranial surface before a 21-gauge guide cannula was positioned 0.8 mm posterior and 1.5 mm lateral to bregma (co-ordinates determined according to Paxinos and Watson 1986). The guide cannula was inserted to a depth of 3 mm through a 1.5 mm diameter hole drilled in the cranium. It was fixed to the skull with acrylic dental cement (Simplex Rapide; Austental Dental, UK) adhered to two jeweller's screws (4 mm long x 2 mm diameter) positioned anterior and posterior to the cannula. Once the dental cement was dry, the skin around the cannula was sutured (size 3/0 mersilk, Ethicon, Johnson and Johnson International, USA). For post-operative analgesia, rats were injected with 10 µl/kg buprenophrine (Vetergesic, Reckitt Benckiser Healthcare, Hull, UK) and a bolus intramuscular injection of saline to aid recovery. Animals were allowed to recover for one week and during this time rats were given food and water *ad libitum*.

3.3.4 Fos immunocytochemistry following i.c.v. histamine administration or i.p. administration of a H_3R agonist and H_3R inverse agonists

I.c.v. histamine administration or i.p. administration of thioperamide, imetit or NNC1202 was repeated in animals that had not been fasted overnight, in order to examine brain areas activated. On the day of the experiment, rats were administered with histamine or isotonic saline (2 µl injection volume) i.c.v., or with the thioperamide, imetit, NNC1202 or isotonic saline injections (1ml/kg) i.p. After the injection, the cannulae were checked to ensure reflux did not occur. 90 min following injection, the rats were deeply anaesthetised with sodium pentobarbitone (100 mg/kg (B. Braun, Sheffield, UK) and perfused transcardially with heparinised (10,000 I.U./l, Leo Pharma, Denmark) isotonic saline (0.9 % NaCl, B. Braun, Sheffield, UK) for 8 min, followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (PB) for 15 min. Perfused brains were post fixed and cryoprotected in the same fixative with 15 % sucrose added, followed by immersion in 30 % sucrose in 0.1 M PB. 30 µm coronal sections throughout the rostro-caudal extent of the brain were cut using a freezing-sledge microtome and collected into 0.1M PB. Endogenous peroxidase activity was deactivated by incubating sections in a 1.5 % hydrogen peroxide, 20 % methanol and 0.2 % Triton X-100 in 0.1 M PB for 30 minutes at room temperature, followed by three 10 min washes in 0.1 M PB. To block non-specific staining, slices were incubated for 1 hour at room temperature in 2 % normal goat (NGS) blocking serum (0.1 M PB, 0.3 % Triton X-100, 2 % NGS). Slices were then incubated for ~24 hours at 4°C in goat anti-rabbit c-Fos antibody (SC052, Santa Cruz Biotechnology Inc., USA) diluted 1:1000 in the NGS blocking serum. Excess antibody was removed by washing sections three times in 0.1 M PB with 0.3 % Triton X-100 before the sections were incubated in goat anti-rabbit immunoglobulin (IgG) peroxidase complex (Vector Laboratories Inc., USA) diluted 1:500 in blocking serum.

Following three washes in 0.1 M PB the slides were incubated for 1 ½ hours at room temperature in streptavidin-biotinylated horseradish peroxidase complex (source) 1:400 in 0.1M PB. Following three further washes in 0.1 M PB, the bound antibody-peroxidase complex on the sections was visualised using nickel-intensified diaminobenzidine (DAB). Sections were washed in 0.1 M acetate buffer (1.64 % sodium acetate, adjusted to pH 6 with glacial acetic acid) incubated in nickel-DAB solution (5 % nickel sulphate in 0.1 M acetate buffer, 0.25 mg/ml DAB, 0.4 % glucose, 0.08 % ammonium chloride and 0.003 % glucose oxidase). The reaction was followed using a microscope and terminated by rinsing the sections in acetate buffer, followed by 0.1 M PB three times, once a strong black precipitate was formed.

Sections were mounted from distilled water onto glass microscope slides using a xylene-based mountant (Ralmont, BDH UK), left to dry and observed using a microscope.

3.3.5 Analyses

Neurones were determined to be c-Fos positive if their nuclei were stained a dark black colour. A qualitative analysis of the whole brain was made to determine regions of interest (i.e. that contained significant c-Fos staining). Regions of interest were then counted bilaterally for each tissue section with the observer blinded to the treatment group. The average number of cells per section was calculated for each animal and averaged to provide a treatment group mean. All sections collected were analysed and each section is 120 µm further than the last. One way analysis of variance (ANOVA) with Bonferonni *post hoc* test was carried out to determine statistical significance between saline and histamine treated animals. Two way ANOVA with Bonferonni *post hoc* test was carried out

to determine statistical significance between saline treated animals and imetit, thioperamide or NN1202 treated animals.

3.5 Results

Examining the brains collected from each of the drug treatments, we found that all had induced c-Fos. There was not a global activation of the brain, but instead, activity was distributed within clearly-identifiable regions. The staining patterns of c-Fos were found to be similar for the different drug treatments and, therefore, these regions of interest were focused on in our quantitative analyses. The regions of interest were the paraventricular nucleus (PVN), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), arcuate nucleus (ARC) in the hypothalamus, anterior hypothalamic area (AHC), lateral hypothalamic area (LH), medial amygdala (AMYm), anterior amygdala (AMYa), central amygdala (AMYc), tuberomammillary nucleus (TM) and the dorsal raphe nucleus (DR). As all staining patterns were similar for histamine and all the drug groups a example of the staining for only the histamine versus control group have been illustrated.

3.5.1 The effect of i.c.v. injections of histamine on neuronal activation

Examination of brains showed that animals that received i.c.v. injections of 200 nmol histamine displayed the induction of c-Fos. Four hypothalamic nuclei known to be involved in feeding and appetite had high numbers of c-Fos-positive neurones. These included the PVN, VMN, DMN and the ARC. We found that these areas had significantly increased numbers of c-Fos-positive neurones in rats that received histamine, compared with control rats (figure 3.1). For the PVN there were a high number of c-Fos-positive cells per section (50 ± 5) compared

with the control rats (7 ± 2; $P < 0.0001$). The PVN was seen to have a high number of c-Fos-positive cells that were close together and with nuclei of varying sizes suggesting different types of neurone and potentially non-neuronal cells were activated in this area (figure 3.2). The VMN had significantly more c-Fos-positive cells in animals that received i.c.v. histamine injections (27 ± 4) compared with control animals (2 ± 1, $P < 0.0001$) (figure 3.1). The DMN had a large number of c-Fos-positive cells in the histamine-treated rats (120 ± 20) compared with vehicle-treated animals (46 ± 20, $P < 0.0001$) (figure 3.1). These cells were more spaced out throughout the DMN rather than a close group of cells as seen in the PVN. The ARC also had increased number of Fos-stained neurones in rats that received histamine injections (30 ± 3) compared with those that received saline vehicle (9 ± 3; $P < 0.0005$) (figure 3.1). The TM was found to have an increased number of c-Fos-positive cells in the brains collected from rats that received histamine injections compared with those that received vehicle. There were 50 ± 12 c-Fos-positive cells per section found within the histamine-treated animals with only 12 ± 5 c-Fos-positive cells being found in the saline-treated control animals ($P < 0.05$) (figure 3.1).

There was also a high amount of staining within the central amygdala (AMYc) of rats treated with histamine. Rats treated with histamine were found to have significantly higher neuronal activation (45 ± 29) as compared with control rats (12 ± 9, $P < 0.05$) (figure 3.1). These c-Fos-positive cells were tightly grouped together suggesting they could be a specific population of cells within the AMYc. The other areas of the amygdala did not show significant differences between histamine and control groups.

After examining the DR of both the histamine- and saline-treated animals we counted a significant difference between numbers of neurones activated. The saline-treated animals had a much lower level of staining than the histamine-treated group (figure 3.1). The histamine-treated group had an average of 121 ± 5

positive cells per section whereas as the saline-treated group had 46 ± 5 (P < 0.05) c-Fos positively stained cells.

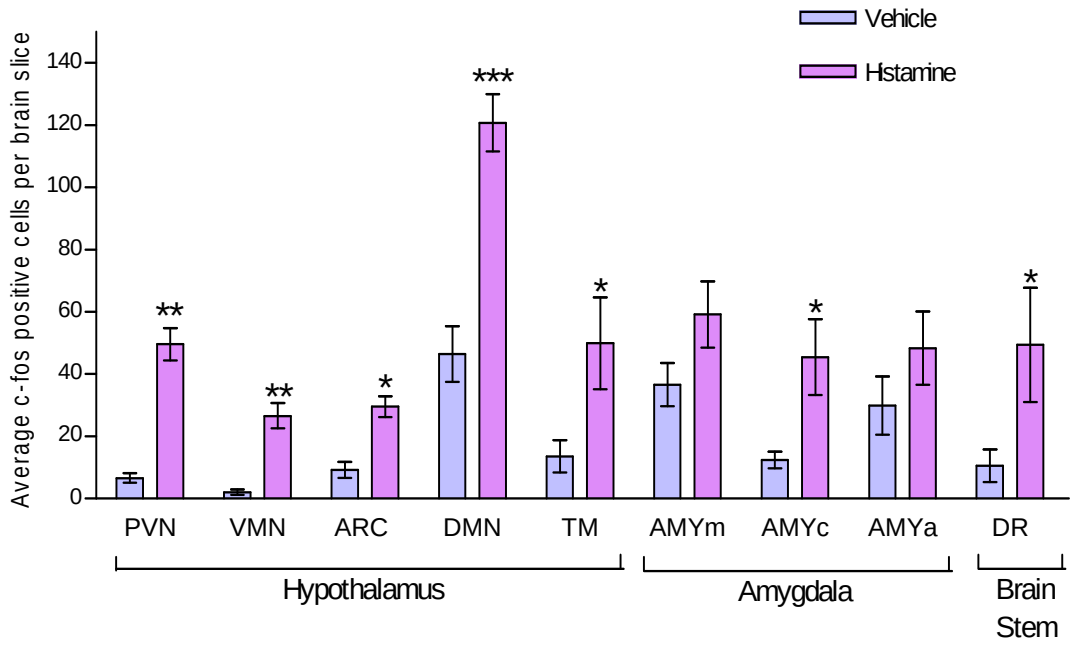

Figure 3.1.; The effect of histamine administration on c-Fos immunoreactivity in the rat brain. Animals received an injection of histamine (200 nmol) or saline into the intracerebral ventricle 90 minutes before transcardial perfusion. Data are expressed as mean ± SEM. Two way ANOVA with Bonferonni *post hoc* test was carried out to determine statistical significance, * P < 0.005, ** P < 0.0005, *** P < 0.00005.

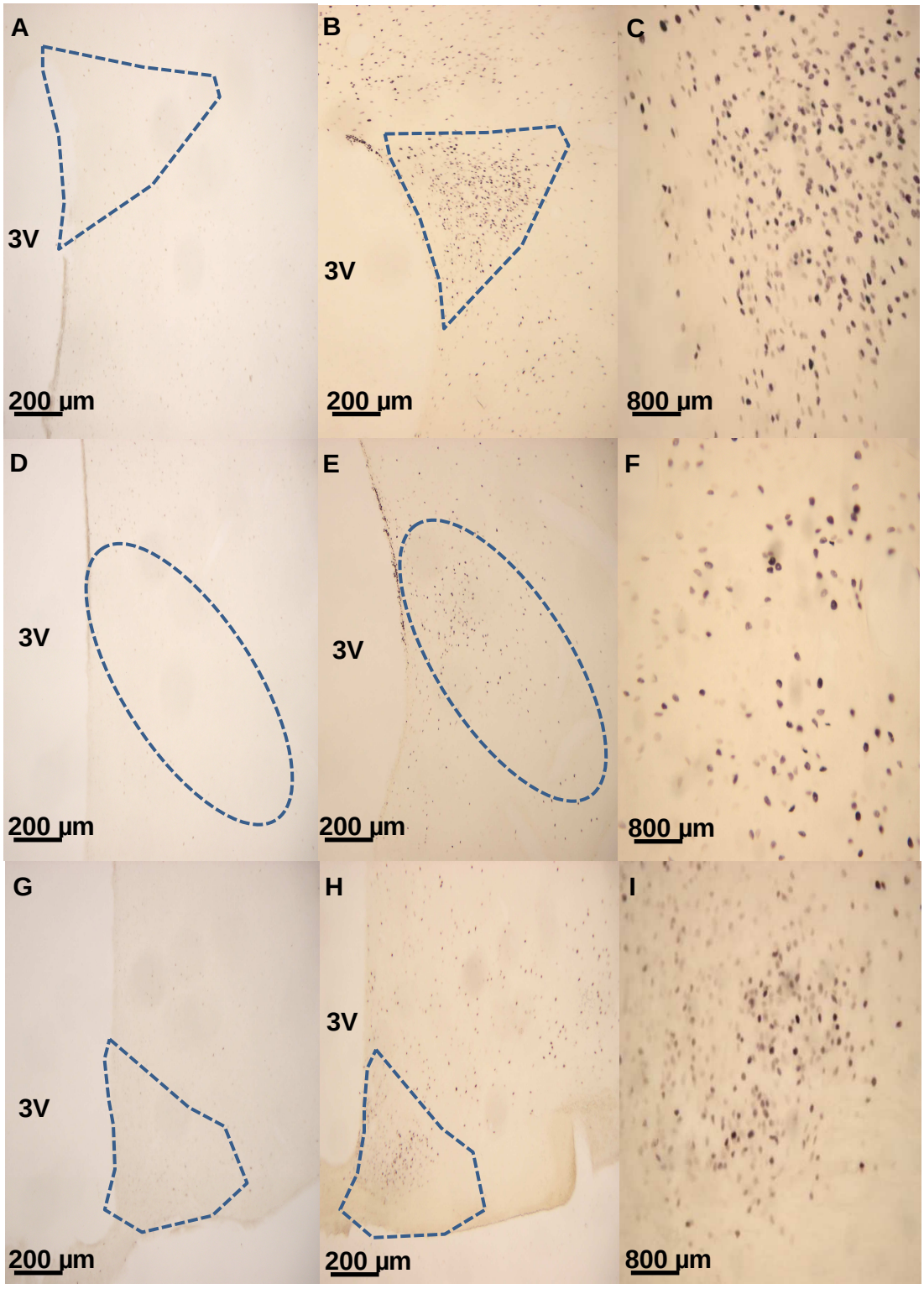
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Figure 3.2. (see previous page)**;** Representative photomicrographs of regions of interest known to be integral in appetite control that display significantly high level of activation following histamine administration. Animals received an injection of histamine (200 nmol) or saline into the intracerebral ventricle 90 minutes before transcardial perfusion. A, D & G represent saline treated animals, B, C, E, F, H & I represent histamine treated animals. A – C illustrates c-fos staining in the PVN, D - F represents c-fos staining in the VMN and G – I represents c-fos staining in the ARC. 3V = third ventricle. A similar a pattern of staining was also observed in these areas for each of the H_3R compounds tested.

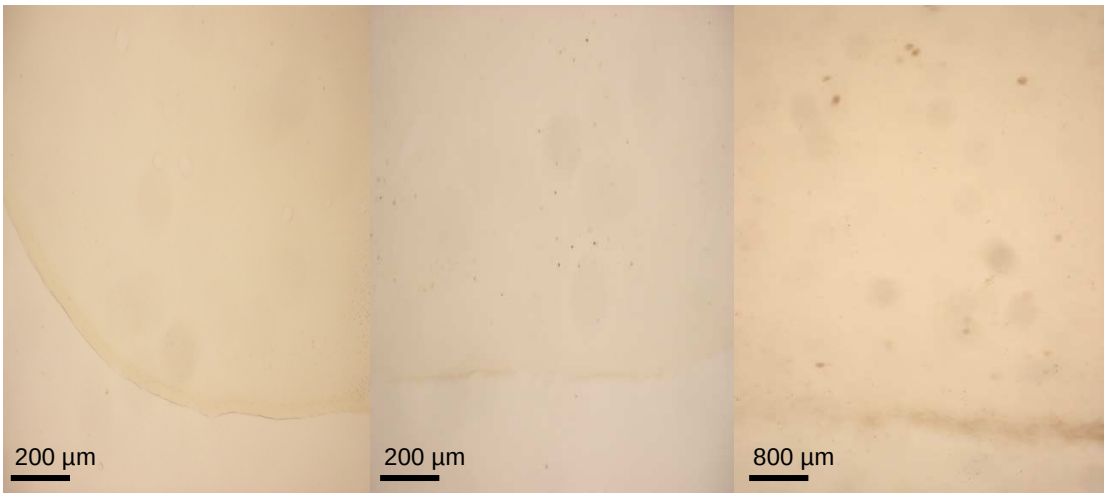


Figure 3.3.; Representative photomicrographs of the cortex of saline treated and histamine treated animals. These photomicrographs illustrate that c-fos staining was not universally found in all regions throughout the brain but was specific to certain brain areas. In particular those regions of interest known to be integral in appetite control. Animals received an injection of histamine (200 nmol) or saline into the intracerebral ventricle 90 minutes before transcardial perfusion. A. Represents animals that received an injection of saline, B & C represent a animal that received a injection of histamine. A similar a pattern of staining was also observed in these areas for each of the H_3R compounds tested.

3.5.2 The effect of i.p. injections of H_3R compounds on neuronal activation

I.p. injections of 10 mg/kg imetit, 2 mg/kg thioperamide or 30 mg/kg NNC1202 significantly increased c-Fos-positive neurones in a number of areas within the hypothalamus compared with control rats (figure 3.4). Overall there were also similar patterns of c-Fos staining between the separate drug groups. A significantly higher number of cells were stained for c-Fos within the DMN of imetit-, thioperamide- and NNC1202-treated animals (99 ± 9, 88 ± 14, 90 ± 9, respectively; $P < 0.001$ for all groups) compared with saline-treated rats (51 ± 6) (figure 3.4). The VMN also had a large number of c-Fos-positive cells within the brains of imetit-, thioperamide- and NNC1202-treated animals (32 ± 4, 36 ± 3, 44 ± 4, respectively; $P < 0.001$ for thioperamide and NNC1202, $P < 0.01$ for imetit), with a significantly lower number within the brains of animals that received saline injections (15 ± 3) (figure 3.4). There were also differences in neuronal activation within the PVN between the drug and vehicle groups. The H_3R agonist, imetit, and H_3R inverse agonists, thioperamide and NNC1202, treated brains were found to have a significantly increased number of c-Fos positive cells (78 ± 16, 126 ± 12, 120 ± 9, respectively; $P < 0.05$ for all groups) compared with the saline-treated group (26 ± 3) (figure 3.4). The ARC was also an area that we discovered differences between the H_3R agonist-, the H_3R inverse agonist- and the saline-treated-controls (figure 3.4). The imetit-treated animals had 49 ± 7 c-Fos-positive cells whereas those that received saline had significantly less cells stained for c-Fos (26 ± 3; $P < 0.001$). The rats that received thioperamide and NNC1202 also had significantly higher c-Fos counts (33 ± 5, 56 ± 3, $P < 0.001$). Interestingly, the H_3R antagonists were less potent at causing neuronal activation that the H_3R agonist within the ARC.

Two other areas within the hypothalamus were also seen to have differences in neuronal activation between drug-treated and vehicle-treated groups. These areas included the AHC and the LH (figure 3.4). The AHC of

control animals had 48 ± 19 c-Fos-positive cells whereas those that received imetit, thioperamide and NNC1202 showed a dramatic increase in neuronal activation, with, 184 ± 25, 147 ± 7, 158 ± 16 ($P < 0.001$) c-Fos-positive cells, respectively (figure 3.4). H_3R agonist- and H_3R inverse agonist-injected animals were also seen to have an increase in neuronal activation within the LH. c-Fos-positive cells were more numerous within the LH in imetit, thioperamide and NNC1202 treated animals (170 ± 14, 146 ± 8, 124 ± 14**,** respectively; $P < 0.001$), than in saline control animals (57 ± 14), suggesting all three drugs cause a increase in neuronal activation in this area. The TM, the source of histaminergic neurones, was also found to show differences in activation (figure 3.4). Brain slices from rats that were treated with saline had 23 ± 6 c-Fos-positive cells per section, whereas those that received imetit injections had 93 ± 6 c-Fos-positive cells ($P < 0.001$), those that received NNC1202 showed 100 ± 10 ($P < 0.001$) cells positive for c-Fos and those that received thioperamide were calculated to have 107 ± 22 c-Fos-positive cells ($P < 0.001$). Thus, all three treatments caused a significant increase in neuronal activation with increased numbers of c-Fos-positive cells compared with saline controls in much a similar way seen when rats received a histamine injection (for comparison see figure 3.2).

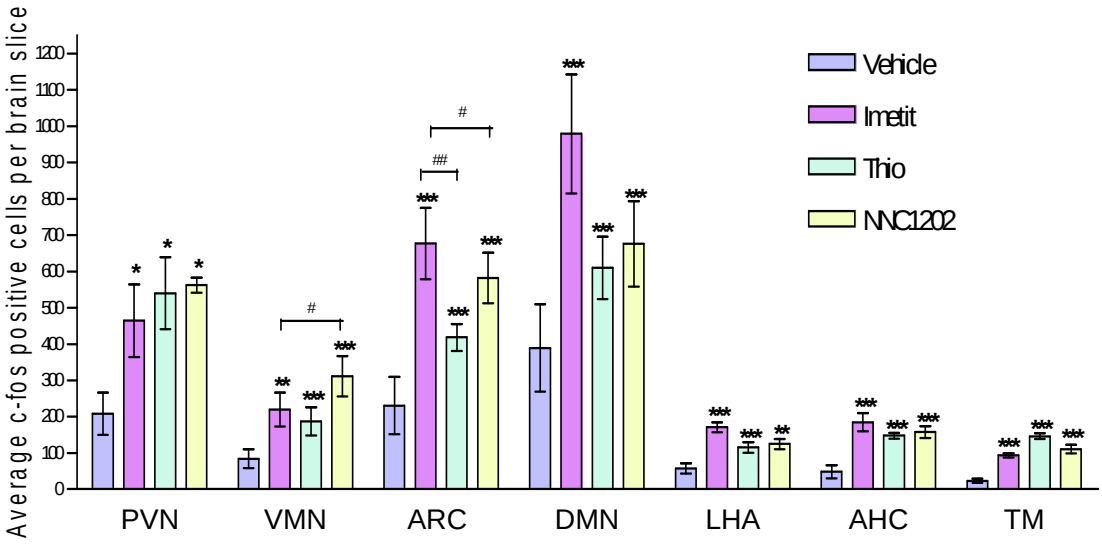


Figure 3.4.; The effect of the H_3R agonist imetit and the H_3R inverse agonists, thioperamide and NNC1202 administration on c-Fos immunoreactivity in the hypothalamus of the rat brain. Animals received an injection of imetit (10 mg/kg), thioperamide (2 mg/kg) or NNC1202 (30 mg/kg) or saline into the intraperitoneal cavity 90 minutes before transcardial perfusion. Data are expressed as mean ± SEM. One way ANOVA with Bonferonni *post hoc* test was carried out to determine statistical significance. * indicates change from control animals. # indicates change from different drug group. # / * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The AMY was another area seen to have variances in neuronal activation between the drug- and saline-treated groups (figure 3.5). The AMYm showed a significant increase in c-Fos-positive cells in brain sections from animals that received imetit (214 ± 20, $P < 0.01$), thioperamide (231 ± 18, $P < 0.01$) and NNC1202 (281 ± 45, $P < 0.001$), compared to those that received saline (77 ± 15) (figure 3.5). The pattern of staining here was evenly spaced out, covering the entire AMYm. The AMYa showed a similar pattern of staining for c-Fos-positive cells. There was a significant increase in c-Fos-positive cells in animals treated with imetit (235 ± 19, $P < 0.001$), thioperamide (163 ± 12, $P < 0.01$) and NNC1202 (151 ± 10, $P < 0.01$), compared with control animals (81 ± 16) (figure 3.5). Again the staining pattern was seen to be evenly spread throughout the entire AMYa. The cells stained positive for c-Fos showed a much more compact pattern in the AMYc compared to the other areas of the AMY (figure 3.5). The AMYc from animals that received imetit and thioperamide showed a significant increase in c-Fos positive cells (187 ± 16, 200 ± 33, respectively; $P < 0.001$) compared with saline controls (40 ± 5) (figure 3.5), suggesting this area was activated due to the effects of the drug. The NNC1202 treatment however showed no significant difference in neuronal activation with only 68 ± 5 c-Fos positive cells being detected. This suggests that the NNC1202 compound does not cause activation within the AMYc, despite the H_3R inverse agonist thioperamide showing a significant increase in neuronal activation in this area.

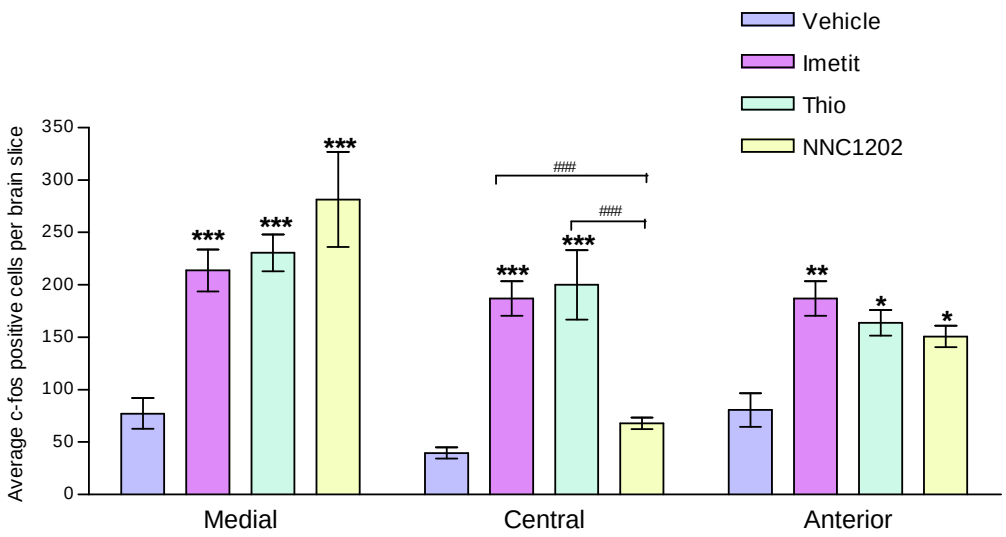


Figure 3.5.; The effect of the H_3R agonist imetit and the H_3R inverse agonists, thioperamide and NNC1202 administration on c-Fos immunoreactivity in the amygdala regions of the rat brain. Animals received an injection of imetit (10 mg/kg), thioperamide (2 mg/kg) or NNC1202 (30 mg/kg) or saline into the intraperitoneal cavity 90 minutes before transcardial perfusion. Data is expressed as mean ± SEM. One way ANOVA with Bonferonni *post hoc* test was carried out to determine statistical significance. * indicates change from control animals. # indicates change from different drug group. * $P < 0.05$, ** $P < 0.005$, ### / *** $P < 0.0005$.

The dorsal raphe nucleus (DR) contained a significantly increased number of c-Fos-positive cells in brain slices from rats that had been treated with imetit, thioperamide and NNC1202 (81 ± 6, 115 ± 15, 110 ± 12, respectively; $P < 0.05$, $P < 0.05$, $P < 0.01$ respectively), compared to rats that had received saline (35 ± 9) (figure 3.6). This staining was seen in more anterior DR areas, with the amount of neuronal activation decreasing more caudally.

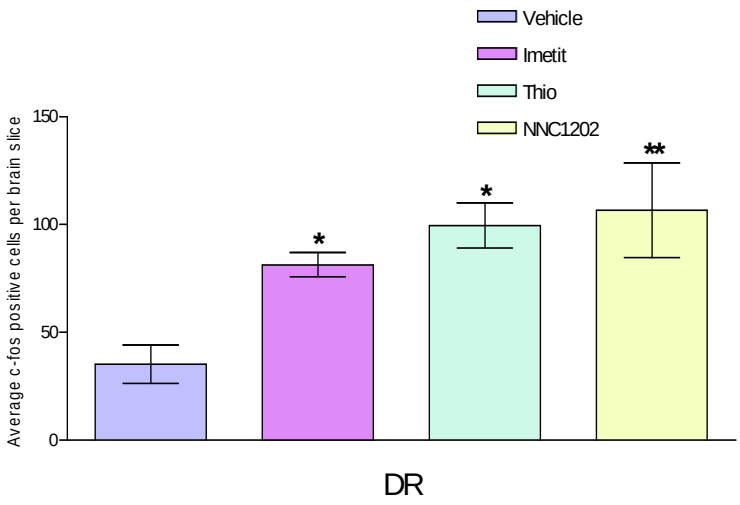


Figure 3.6.; The effect of the H_3R agonist, imetit, and the H_3R inverse agonists, thioperamide and NNC1202, on c-Fos immunoreactivity in the dorsal raphe nucleus (DR) of the rat brain sections. Animals received an injection of imetit (10 mg/kg), thioperamide (2 mg/kg) or NNC1202 (30 mg/kg) or saline into the intraperitoneal cavity 90 minutes before transcardial perfusion. Data is expressed as mean ± SEM. One way ANOVA with Bonferonni *post hoc* test was carried out to determine statistical significance. * $P < 0.05$, ** $P < 0.005$.

3.6 Discussion

We found that Fos was induced only in certain areas within the rat brain (see comparison between figure 3.2 and figure 3.3). Thus, there was no blanket effect of neuronal activation therefore suggesting only certain areas are activated upon administration with histamine. Presence of Fos shows which areas of the brain have been activated but not the process through which it occurs (for example, directly or indirectly). Although c-Fos shows certain areas of the brain that are activated at the time of brain collection it gives no indication if histamine is actually being released endogenously in these areas. Also, c-Fos immunopositive neurones are found in areas that have been activated but this can be due to excitation or a disinhibition (that is due to the local inhibition of otherwise inhibitory neurone/inputs).

3.6.1 Histamine- and H_3R receptor drug-induced neuronal activation within the hypothalamus

Histamine neurones project from the TM to all areas of brain, with the densest projections to nuclei in the hypothalamus. Thus, one might have predicted, a high level of neuronal activation, though not an exclusive activation in these areas. Although, it is noteworthy to add that the activation was confined mainly to areas involved in the regulation of appetite. It is well established that the hypothalamus is essential for controlling appetite (see review King 2006). In particular the VMN, PVN, ARC, LH and DMN play pivotal roles in regulating food intake. We determined that the numbers of c-Fos-immunopositive neurones in the VMN, PVN, ARC, LH and DMN were significantly higher in rats that received an i.c.v. injection of histamine, or i.p. injections of the H_3R agonist, imetit, or the H_3R inverse agonists thioperamide and NNC12302.

The hypothalamus is crucial in appetite control with the VMN, PVN, LH, ARC, DMN and SCN nuclei all playing pivotal roles. Grouped together these areas form complex networks that regulate energy intake and expenditure (Beck *et al.,* 2000). For example, the VMN has direct links with the PVN and DMN and via these it connects indirectly with the LH (Harrold *et al.,* 2004) illustrating the complex circuitry involved in regulating food intake within the hypothalamus. All the hypothalamic areas we found to have increased numbers of c-Fos-immunopositive neurones are known to play important roles in regulating food intake. The VMN and PVN are satiety centres and the LH is a feeding centre and, thus, inform the brain when the animal is full or hungry, respectively. The ARC has a high co-expression of the orexigenic peptides NPY and AgRP (Dhillo, 2007) and also expresses a large population of anorectic signalling cells (Elias *et al.,* 1998) and therefore is extremely important in regulating both hunger and satiation in the animal. These orexigen- and anorexigen-producing neurones project from the ARC onto the VMN, DMH, LH and PVN (Harrold JA., 2004), again illustrating

the complex appetite regulating networks within the rodent brain. Within the DMH, microinjection of orexigenic compounds elicits feeding (Li *et al.,* 1998), and the inhibition of NPY-induced feeding, enhances neuronal c-Fos (Yokosuka *et al.,* 1998). DMH efferents project to both the VMN and PVN, and the DMH receives prominent ARC input. Studies have shown that high densities of c-Fos-positive neurones have been shown within the PVN in response to administration of both orexigenic and anorexigenic signal molecules (Dhillo, 2007). All these areas were shown to increase their neuronal activation in response to histamine, imetit, thioperamide or NNC1202 application suggesting their involvement in controlling food intake at least in part through the histaminergic system.

Fos induction within the hypothalamus might be due to direct activation by histamine itself through the activation of H_1Rs. H_1Rs are located throughout the rodent brain but are known to be high in density in the hypothalamus, including discrete neurones involved in regulating food intake, such as in the VMN and PVN (Sakata *et al.,* 1988). Morimoto *et al* (2001) and Masaki *et al.* (2006) suggest that histamine acts on the H_1R in the VMN and PVN to decrease food intake. As the H_1R is distributed throughout the hypothalamus, histamine may also be acting directly within the ARC, LH and/or DMN. The H_1R causes a reduction in food intake when activated, so administering histamine could have caused an activation of the H_1R within these feeding centres of the rat brain, thus causing the reduction in food intake observed in previous experiments. This also applies to the H_3R inverse agonists, as their actions would have led to the inactivation of the H_3R's ability to regulate the release of histamine and thus resulting in a increase in neuronal histamine with would than cause the same effects seen on food intake or neuronal activation witnessed when histamine itself was administered.

A significantly high number of c-Fos-immunopositive cells were also evident within the TM of the rat hypothalamus. This is not surprising as the TM is the sole

origin of the histaminergic neurones. Also, accumulating evidence suggests histaminergic neural circuits arising within the TM and projecting into the satiety centres of the hypothalamus participate in regulation of energy homeostasis. For example, histaminergic neurones have been found to project from the TM the PVN and VMN. Here the anorectic effect is again thought to be mediated by postsynaptic histamine H_1R. But the density of the H_1R, together with H_3R-mediated control of endogenous histamine concentrations is thought to be crucial for anorectic potency (Malmlof *et al.,* 2005). H_3Rs are present on the cell bodies of the histamine neurones in the TM (Brown *et al.,* 2001). Thus, histamine and the H_3R drugs could be directly acting upon these histaminergic cell bodies causing neuronal activation within this area and this would induce Fos. Equally the histamine may activate H_1R within or around the TM causing the increase in c-Fos-immunopositive neurones being seen. Also, histamine, imetit, thioperamide or NNC1202 could be acting upon the histaminergic nerve endings from the TM. This itself may not induce Fos, but the H_3R inverse agonists may block the ability of the H_3 autoreceptor to negatively regulate the release and synthesis of histamine thus resulting in an inevitable increase of endogenous circulating histamine. This increase in endogenous histamine could be binding to the H_1R in the hypothalamus causing an increase in neuronal activation. As all the substances tested could be acting through the H_1R by altering histamine levels, this could explain the similar patterns of Fos staining seen after the application of histamine and the H_3R agonist and inverse agonists suggesting similar neuronal activation occurs.

The H_3R agonist imetit increased neuronal activation within the same areas as histamine and the H_3R inverse agonist even though it would inhibit histamine release and thus be unlikely to be activating H_1R in these areas. Therefore, imetit could have activated H_3R present or that project to these regions. Alternatively, the H_3R agonist might have acted on H_3 heteroreceptors

and affected the release of other transmitters which in turn is causing an increase in c-Fos staining due to neuronal activation in this area. This applies to the H_3R inverse agonists as well who could have equally been activating H_1Rs via H_3 autoreceptor directed histamine release, or directly activating H_3 heteroreceptors present on other transmitter terminals.

3.6.2 Histamine and H_3R-drug induced neuronal activation within the medial, central and anterior amygdala

Another area showing a significant increase in neuronal activation after histamine or the H_3R drug application was the amygdala. The medial, central and anterior amygdala all showed increased neuronal activation. Krettek *et al.* (1978), illustrated how individual amygdalar cell groups in rat project to the hypothalamus. This work revealed that the medial, basomedial, and posterior amygdalar nuclei project to the VMN, that these same nuclei project to the premammillary nuclei, and that the central and basolateral nuclei also project sparsely to the rostral lateral hypothalamic zone (Petrovich *et al.,* 2001). Central amygdala lesions robustly potentiate feeding. Thus, activation in this area might be involved in the sharp reduction in feeding seen. In addition, the amygdala has anatomical connections with neural circuits in the hypothalamus that are known to control feeding behaviour (Swanson LW., 2000, Petrovich *et al.,* 2001).

The H_1R is expressed abundantly in the amygdala (Haas *et al.,* 2003) and, thus, could have been activated after the application of histamine. The H_3R inverse agonists would have also increased endogenous histamine, which could have then acted upon the H_1R. These connections and the presence of H_1R could explain why significant c-Fos staining was observed within the amygdala after histamine administration and the injections of the H_3R inverse agonists, thioperamdie and NNC1202.

The H_3R agonist imetit was also proven to increase neuronal activation and although it would result in a reduction in histamine, it could have, like the H_3R inverse agonist drugs, activated H_3Rs that are present or project to the amygdalar regions. Alternatively, all three compounds might have acted on H_3R heteroreceptors. Imetit, thioperaminde or NNC1202 could have acted on H_3R heteroreceptors, such as those that have been found on glutamatergic terminals. There are a large number of glutamate neurones within the amygdala which have been shown to project onto the LH (Swanson *et al.,* 1998). Thus, our H_3R drugs may be affecting the release of glutamate within the amygdala causing an increase in c-Fos staining due to neuronal activation in this area.

3.6.3 Histamine- and H_3R- drug induced neuronal activation within dorsal raphe nucleus

The dorsal raphe nucleus (DR) also had an increase in c-Fos-positive cells after both histamine and H_3R drugs were given. H_1R rather than H_3R have been found within the DR (Barbara *et al.,* 2002) and, thus, could again be activated by the increase in endogenous histamine either after the administration of histamine or the H_3R inverse agonists.

There is a large serotonergic neurone population within the DR (Barbara *et al.,* 2002). Serotonin is involved in the control of feeding behaviour (Blundell JE., 1977, Simansky KJ., 1996, Kaye *et al.,* 1998, Schuhler *et al.,* 2005) and is especially linked to hunger and satiety (Blundell *et al.,* 1987). Serotonergic transmission from the DR (Fletcher *et al.,* 1993; Ohliger-Frerking *et al.,* 2002) to the VMN has been proven to regulate feeding (Fernández-Galaz *et al.,* 2010). H_3R, when acting in their heteroreceptor form, can control the release of serotonin by acting presynaptically (Schlicker *et al.,* 1988, Threlfell *et al.,* 2004). For example, thioperamide has been shown to increase serotonin release (Threfell *et*

al., 2004). Also a number of atypical H_3R antipsychotic drugs affect food intake and appetite by influencing serotonin release via the H_3R. Thus, the H_3R drugs could have been not only affecting histamine release and synthesis but also other neurotransmitter release such as serotonin caused an increase in neuronal activation and hence shown a significant increase in Fos production in this area.

To conclude we have demonstrated that only specific areas within the rat brain are activated upon receiving the compounds tested. We have shown some areas within the rodent brain histamine, imetit, thioperamide and NNC1202 act upon, and have determined that each drug group produces very similar patterns in neuronal activation. In particular, we have illustrated that all the known key feeding or satiety centres showed a significant increase in c-Fos-immunopositive neurones, suggesting activation of these areas by histamine and the H_3R drugs is causing the changes on food consumption that these drugs exhibit.

Chapter 4:

Modulation of neuronal activity within the VMN by histaminergic receptor ligands

4.1 Introduction

4.1.1 The VMN and appetite

For a number of decades, discrete nuclei in the basal hypothalamus have been accepted as crucial in the regulation of daily energy homeostasis, especially those sites connected with neural mechanisms affecting appetite and energy homeostasis (Kalra *et al.,* l999). These hypothalamic sites include the VMN, DMN, PVN, and LH. The belief these regions contained neurones affecting ingestive behaviour was based on the results of numerous lesions studies or surgical transections of neural pathways in these hypothalamic regions (Anand *et al.,* 1951; Bray GA 1984). With the exception of lesions in the LH, these experimental manipulations within the brain disrupted the daily food intake pattern to produce permanently enhanced hyperphagia, suggesting appetite-regulatory mechanisms may be confined to a small number of morphologically well-defined regions within the hypothalamus (Kalra *et al.,* 1999). The VMN is one of the most important sites known to regulate appetite and food intake and is commonly referred to as the satiety centre. The satiety centre hypothesis was first proposed in 1950 (Kennedy GC., 1950.). Here Kennedy (1950) suggested that the VMN was the brain's satiety centre, as when active, feeding behaviour is inhibited and when disrupted or ablated feeding greatly increases, indicating its main role is to tell the brain when the body is full.

There is evidence that suggests the VMN is receptive to several appetite-regulating signal molecules, including orexigenic and anorexigenic signals. For example, microinjection into the VMN of NPY (Stanley *et al.,* 1985), galanin (Kyrkouli SE., 1990; Kyrkouli *et al.,* 1986; Schick *et al.,* 1993), GABA (Grandison *et al.,* 1977; Kelly *et al.,* 1979), and β-endorphin (Grandison *et al.,* 1977) all stimulate hyperphagia, whereas injections of leptin reduces feeding (Munzberg H., 2010; Velkoska *et al.,* 2003). This suggests that receptors for each of these

signals exist in the VMN and each of these compounds have valuable roles in appetite and feeding control. Further, the VMN is neurally linked with several hypothalamic sites implicated in the control of ingestive behaviour.

Brobeck (Brobeck JR 1956) and Kennedy (Kennedy GC 1950) observed that even small lesions around the ventrolateral borders of the ventromedial hypothalamus and extending to the base of the brain can have affects on feeding behaviours. Anand and Brobeck (Anand *et al.,* 1951) later reported that obesity could be produced by either ventromedial hypothalamus lesions or by small lesions just lateral to the ventromedial hypothalamus. Lesions posterior to the ventromedial hypothalamus and dorsolateral to the mammillary body also cause obesity (Graff *et al.,* 1962). Although researchers have observed weight gains in rodents with lesions just ventrolateral or just posterior to the ventromedial hypothalamus, lesions directed at the VMN cause the greatest obesity (King BM., 2006). Ventromedial hypothalamus lesion-induced hyperphagia and obesity have been observed in a variety of species besides rats, including ground squirrels, mice, dogs, cats, pigs, monkeys and even birds (Mrosovsky N. 1974; Romaniuk A. 1962; Rozkowska *et al.,* 1971; Brooks *et al.,* 1946), illustrating the species wide importance of this brain area in the regulation of feeding. Hypothalamic obesity has been documented in humans as well when damage has occurred in the hypothalamic brain area due to injury or other outside causes (Bray *et al.,* 1975). Although, I believe it important to note here that it is difficult to interpret the results of such physical lesions as the damage can be indiscriminate, involving, cell bodies or fibre pathways, and not be specific so that they include other surrounding brain regions.

A number of studies have demonstrated that more selective knock out models or disruption in peptide signalling within the VMN can also cause hyperphagia resulting in weight gain and obesity. BDNF is highly expressed within the VMN, and its expression is dramatically reduced by food deprivation (Xu *et al.* 2003).

Mice with reduced BDNF receptor expression or decreased BDNF signalling significantly increase food intake and thus have elevated body weight (Rios *et al.* 2001; Xu *et al.* 2003). The VMN has also been identified as a key target for leptin, which acts on the hypothalamus to inhibit feeding and stimulate energy expenditure (Dhillon *et al.* 2006). It is also rich in glucose-responsive neurones (GRN) which respond to a rise in blood glucose that may help to cease feeding (Levin *et al.,* 1999; Borg *et al.,* 1995). Zhao *et al.* demonstrated the targeted deletion of the VMN transcription factor steroidogenic factor 1 (SF-1) results in agenesis of the VMN and obesity (Majdic *et al.,* 2002; Zhao *et al.,* 2004). Additionally, SF-1-driven deletion of the signalling form of the leptin receptor, lepr-B, can also results in an obese phenotype (Dhillon *et al.,* 2006). These studies all illustrate the importance of the VMN in controlling food intake and show that not only disruption in the neuronal structure within this area disrupts feeding but also alterations in the signalling of a number of peptides or hormones affecting energy homeostasis regulation.

4.1.2 The VMN and histamine signalling

The rat VMN has long been divided on cytoarchitectonic grounds into ventrolateral, central and dorsomedial parts (Canteras *et al.* 1994). McClellan *et al.* (2006) suggest the VMN contain a bilateral cell group that have an elliptical shape. In coronal sections, the anterior region consists of a circular aggregate of densely packed perikarya, whereas the middle and posterior VMN are formed by two regional cell masses, dorsomedial and ventrolateral, separated by a diagonal cell-poor zone (Canteras *et al.* 1994). The VMN has been thought of as a collection of heterogenous cell types, some of which have been identified but most of which have not (McClellan *et al.* 2006). Cell identity is an important

characteristic of brain organization and the VMN cell population has been somewhat unidentified thus far. Cell sizes have been seen to differ greatly, ranging from between 5 – 20 µm. Sakuma *et al.* (1983) observed differences in the spike duration and amplitude between groups of neurones with different sizes within the VMN. The larger surface area of the larger neurones allows a greater peak current flow, and the action potentials in these cells are finished in a much shorter period. The amplitude of the extracellular spikes has also been shown to vary directly with the transmembrane current flow (Sakuma *et al.* 1983). Thus as a rule, large neurones will generate larger spikes of shorter duration, and their extracellular fields will be detected over great distances (Humphrey, 1978). Two main neuronal types, large and small, exist in the VMN. (Murphy *et al.* 1969; Ono *et al.* 1982). Ono *et al.* (1982) found small neurones are restricted to the medial border of the nucleus. On the other hand, Murphy & Renaud (1969) described a cluster of small neurones at the lateral edge of the VMN. Ono et al. (1982) illustrated the presence of large neurones in the core of the VMN and scattered distribution of small neurones all over the nuclear region. Larger neurones have thicker axons, which conduct action potentials faster than thinner ones (Paintal, 1966). Sakuma *et al.* (1983) found a the lack of any systematic difference in the antidromic spike latency between the two groups of VMN neurones with different sizes suggesting the conduction distance may not be consistent for all VMN neurones. Murphy & Renaud (1969) suggest small VMN neurons are interneurones which transmit inhibitory amygdala effects to the VMN, and they propose the large neurones are the major effector cells of the VMN.

As shown in the previous chapters, there is abundant evidence to support a role of brain histamine in food intake and energy metabolism. An early

iontophoretic:extracellular recording study in anaesthetised rats found that histamine could either increase or depress firing in the VMN, ARC, DMN and LH (Renaud LP., 1976). There is also evidence to suggest histamine acts within the VMN to cause shifts in feeding. In fact, the VMN is reported to be the preferential site of histamine-mediated suppression of food intake in the mammalian brain (Haas *et al.,* 2008). The regional distribution of histaminergic fibres and H_1R is uneven, but is most dense in the hypothalamic nuclei related to food intake, such as the VMN and PVN (Palacios *et al.,* 1981). Ookuma *et al.* (1993) suggest that as the H_1R has a suppressive role on feeding it could be explained by the fact these receptors are distributed more densely in the nuclei involved in the suppressive function of food intake. Thus, histamine, through H_1R, conveys signals for suppression of food intake to the satiety centre in the VMN (Ookuma *et al.,* 1993; Sakata *et al.,* 2003). Evidence for the VMN H_1R suppressive effects on feeding come from studies demonstrating that microinfusion of H_1R antagonists directly into the VMN but not PVN or LH elicits feeding responses and increases both meal size and duration (Fukagawa *et al.,* 1989; Sakata *et al.,* 2003). Likewise, electrophoretic application of H_1R antagonists suppresses the firing of glucose-responsive units in the VMN but not the PVN or LH (Fukagawa *et al.,* 1989). Also, feeding rhythms are disrupted in H_1R-deficient mice (Masaki *et al.,* 2001), and H_1R antagonists given selectively in the VMN but not other regions induces feeding and suppress the firing of glucose-sensitive neurons (Fukagawa *et al.,* 1989; Haas *et al.,* 2008).

There is little or no research suggesting the H_3R is present within the VMN but what is known is that the H_3R can regulate histamine release within the ventromedial hypothalamus through its autoinhibitory pathways. H_3R within or around the ventromedial hypothalamus can alter the release of endogenous histamine locally, thus could be affecting histamine's ability to bind to H_1R within the area and cause changes in appetitive behaviour.

4.2 *Objectives*

4.2.1 How does histamine affect the firing rate of cells within the VMN?

We have determined that histamine can affect the feeding behaviour of rats when administered centrally. We have also demonstrated that injections of histamine and H_3R based drugs increase c-Fos induction, which is a marker of cellular activation, within the feeding centres of the rat brain, including the VMN. The VMN has also been shown by a number of research groups (see Chapter 5 introduction) to not only play a pivotal role in controlling feeding behaviours in the rat but also to be highly innervated by histamine neurones, and to contain H_1R within and around it. Thus, we wanted to determine what was happening at the neuronal level within the VMN. Little research has looked at histamine's affects using the extracellular electrophysiology technique, therefore, our aim was to determine firstly what changes were seen when histamine was introduced to a rat brain slice, and specifically to determine whether histamine had an inhibitory or excitatory affect on the firing rate of cells in this area.

4.2.2 Is the increase in neuronal firing via a H_1R?

Whilst a number of research groups (Palacios *et al.,* 1981; Ookuma *et al.,* 1993; Sakata *et al.,* 2003) suggest the presence of the H_1R within and around the VMN, little has been published about the presence of the H_3R. It is widely accepted that the H_3R can control the endogenous histamine levels within the rat brain and, therefore could possibly affect histamine's release and actions within the VMN. Once we had determined how histamine affected the firing rate of cells within the VMN, we then wanted to determine the role of H_1R and H_3R. To do this we aim to administer H_1R and H_3R drugs to determine how they affect the local actions of histamine.

4.2.3 Are H_3R in the VMN autoreceptors or heteroreceptors?

As mentioned previously, the evidence of H_3R presence within the VMN is scarce. We determined using extracellular electrophysiology that histamine was acting via both H_1R and H_3R within the VMN, thus suggesting the presence of the H_3R in this area. After demonstrating that the H_3R is present within the VMN, we then wanted to determine whether these are H_3 heteroreceptors or autoreceptors. To do this we co-administered an H_1R antagonist after blocking H_3R. If H_3R are autoreceptors, then H_1R antagonism would block the actions of an H_3R inverse agonist. Whereas if H_3R were heteroreceptors, then co-administering an H_1R antagonist would have no additional effect.

4.3 Methods

4.3.1 Animals

Male Sprague-Dawley rats, aged 6-8 weeks (Charles River, UK) were housed within The University of Manchester animal facility in a temperature-controlled room (22°C ± 1°C), under a 12:12 h light/dark cycle (lights on 08.00-20.00), with a relative air humidity of 45 ± 10 % and had free access to food and water (Beekay, UK). All animals were maintained in the facility for at least one week prior to the start of the experiment. These and all other experiments conformed with the Home Office (Animals) Procedures Act (1986) and local ethical review.

Animals were culled using a UK Home Office recommended Schedule 1 procedure (see below).

4.3.2 Drugs

Histamine, thioperamide, pyrilamine and imetit (all Sigma) were the drugs used for this study. All drugs were dissolved in sterile water to stock concentration, aliquoted and stored at -20^{o}C. Immediately before use, an aliquot was thawed, diluted to the appropriate working concentration in artificial cerebrospinal fluid (aCSF) and bath applied to the slice via the perifusion line. Concentrations were determined following review of the literature.

4.3.3 Artificial Cerebrospinal Fluid (aCSF)

2 l of aCSF was prepared fresh each day with the following constituents: NaCl: 124.0 mM; $NaHCO_3$: 25.5 mM; KCl: 3.3 mM; KH_2PO_4: 1.2 mM; $MgSO_4$: 1.0; $CaCl_2$: 2.5 mM and D-glucose; 5 mM (all Sigma). In order to determine whether the response of neurones to peptides was direct, we used a low $CaCl_2$ and high $MgSO_4$ solution to block pre-synaptic activation from other neurones. Only the amount of $CaCl_2$ and $MgSO_4$ was changed, using 0.3 mM and 9.0 nM, respectively. The aCSF was adjusted to pH 7.4 and constantly perifused with 95% O_2/5 % CO_2.

4.3.4 Slice preparation

Rats were killed by cervical dislocation and decapitation under isofluorane anaesthesia. The brain was rapidly removed and dissected to form a tissue block containing the hypothalamus. Coronal brain slices 400 μm thick were cut in ice-cold aCSF using a Vibroslicer (Campden Instruments, Loughborough, UK). Usually, three slices that included the VMN were obtained from the

hypothalamus, and the slices not used immediately were stored in oxygenated aCSF through the day.

4.3.5 Electrophysiological Rig

Brain slices were transferred to a PDMI-2 submerged slice microincubator (Figure 3.1) (Medical Systems Corp., NY, and USA). The slice sits in a culture dish surrounded by a 350 μm thick spacer and is stabilised using a nylon ladder attached to a collar, which sits on the spacer.

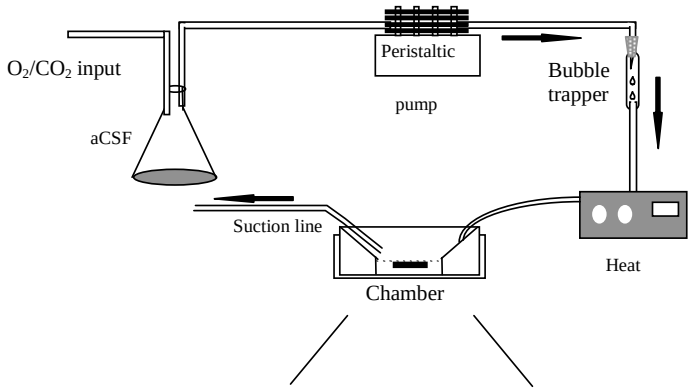


Diagram 4.1.; A schematic representation of the electrophysiological experimental arrangement.
The diagram illustrates the flow of 10mM glucose aCSF, via a peristaltic pump, through the bubble trap, warmed at the heat exchange (via a TC-202 bipolar temperature controller) and superfused over the oxygenated brain slices contained in the PDMI-2 micro-incubator chamber; waste solution is removed via a suction line.
Modified from Forsythe & Coates (1988).

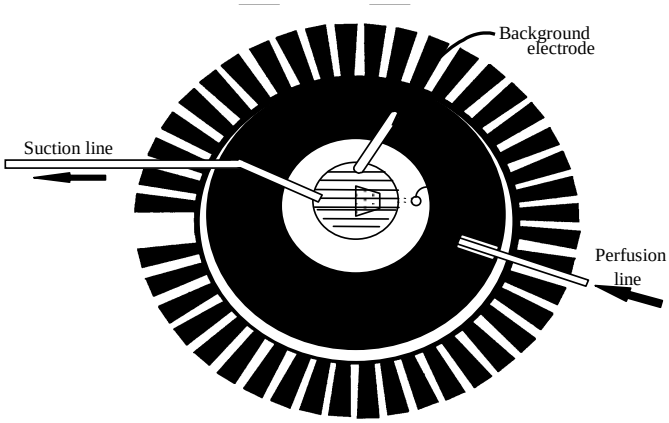


Diagram 4.2.; A schematic representation of the electrophysiology recording chamber.
A top down view of the brain slice recording chamber showing the input locations of the perfusion line, background electrode, suction line, and the orientation of the nylon ladder which stabilises the brain slice and allows unrestricted microelectrode manipulation.
Modified from Forsythe & Coates (1988).

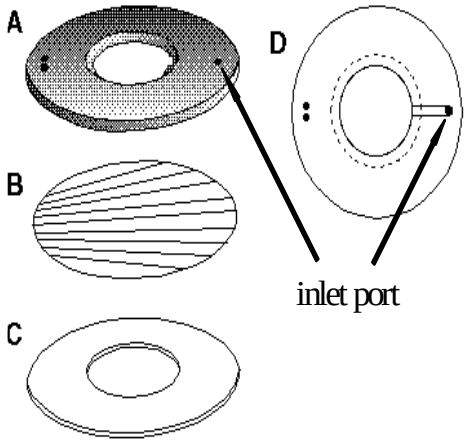


Diagram 4.3.; A diagram of the insert assembly showing the collar (A), the nylon ladder (B) and perspex spacer (C) which sit in the 35 mm tissue culture dish. The underside of the collar (D) illustrates the location of the inlet port.

Slices were maintained for 8-12 h by perfusion (approximately 1.5ml/min) with oxygenated aCSF. The tissue bath and perfusion solutions were warmed to approximately 35°C using a TC-202 temperature controller (Medical Systems Corp.). Slices were allowed to equilibrate for at least 1 h before electrophysiological recordings began.

4.3.6 Electrophysiological Recording

Single unit activity of hypothalamic neurones was recorded extracellularly with borosilicate glass electrodes (Harvard Instruments, Herts, UK) filled with 2 M NaCl (resistance approximately 5 MΩ). Action potential spikes were amplified (× 20,000) filtered (bandwidth 300 Hz to 3 kHz) and visualised using an oscilloscope. Amplification, filtering and spike discrimination were performed using a NeuroLog modular system (Digitimer Ltd, Herts, UK). Data were collected and plotted as integrated histograms on a PC running Spike 2 software (Cambridge Electronic Design, Cambridge, UK). The size of the spikes recorded was between 10-100 mV. Only spikes at least two times the size of the

background noise were recorded and the cell firing rate was recorded for 10 minutes to obtain a stable baseline firing rate, prior to a change in aCSF or drug treatment.

4.3.7 Treatment

Once a stable baseline firing rate was established, the drug was applied via the perifusion line. To identify histamine-responsive neurones, 5 µM histamine was applied for 4 minutes. Normal aCSF was then applied for 20 minutes to allow the histamine to be washed off, before either 20 µM thioperamide or 20 µM pyrilamine was applied for 4 minutes. Again normal aCSF was then applied for 20 minutes to wash off the drug treatment. For experiments using low $CaCl_2$/ high $MgSO_4$, this aCSF was applied for 10 minutes before the drugs diluted in low $CaCl_2$/ high $MgSO_4$ aCSF were applied at the concentrations and times shown above. A return to stable baseline firing was re-established for 10 minutes in low $CaCl_2$/ high $MgSO_4$ aCSF before application of the drug, and then a recovery period of 20 minutes was allowed for the firing rate to return to basal levels before the next drug application.

4.3.8 Identification of Neurones and Analysis

Recordings were only taken from the neurones in the dorsomedial region of the VMN. A VMN neurone was considered to have responded if the firing rate increased or decreased by 20 % relative to the initial 5 min baseline recording, following and during a change in aCSF or drug application. Responses are given as a mean firing rate ± S.E.M. and statistical significance was accepted when $P < 0.05$ using an unpaired t-test.

4.4 Results

4.4.1 Histamine increases neuronal firing rate in the VMN

In this study a total of 197 spontaneously firing VMN neurones were tested for their responsiveness to bath application of 5 µm histamine. The cells showed an average basal firing rate of 1.91 ± 1.73 Hz and a range of 3.55 – 0.97 Hz. 122 out of 197 cells (62 %) showed an increase in firing when histamine was applied, whilst four decreased firing (2 %) and 71 had no response (36 %). Those that increased in firing had an average rate of 3.47 ± 2.01 Hz following application of 5 µm histamine. Those that decreased firing whilst histamine was applied showed an average neuronal firing of 0.62 ± 0.46 Hz. Finally, the 71 cells recorded that were classed as unresponsive to histamine showed an average neuronal firing rate 1.10 ± 1.20 Hz whilst histamine was present. All changes in firing of individual neurones were shown to return to basal levels within 20 minutes post the application of histamine.

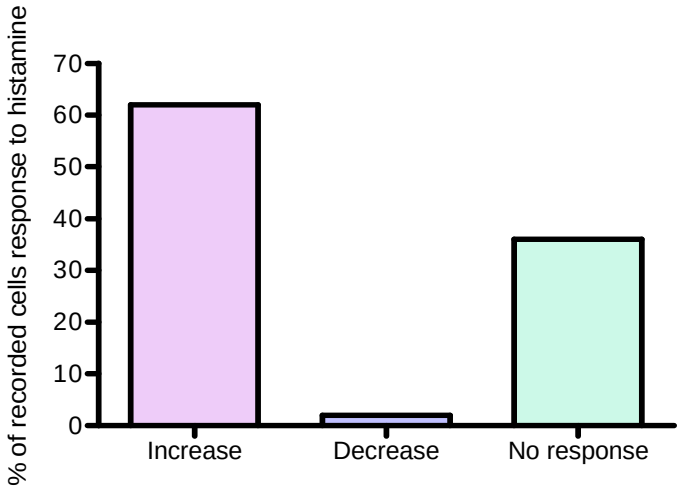


Figure 4.1.; This graph illustrates the differences in the response that cells have to histamine when it is applied to a brain slice *in vitro*. As can be seen the majority of cells showed an increase in neuronal firing when histamine was applied. Around a third of the cells we recorded from were not responsive suggesting around two-thirds are able to respond to histamine, and almost 97 % of these do so with an excitatory response. Only 4 out of 197 cells showed an inhibitory response to histamine.

The increase in neuronal firing seen in 122 of the cells we recorded was very rapid following the introduction of histamine, bearing in mind a slight delay is incurred as the drug is introduced into the bath via the perifusion line (estimated at around 60 seconds). Figure 4.2 shows a typical electrophysiology trace of a VMN neurone responding to histamine application. The increase in firing rate is almost instant and there is a period of constant rapid firing spikes before a graded decrease in response. The neurone takes around 15 minutes to return to basal firing rate. The return to its basal firing rate indicates that the neurone has not been irreversibly altered by the application of histamine.

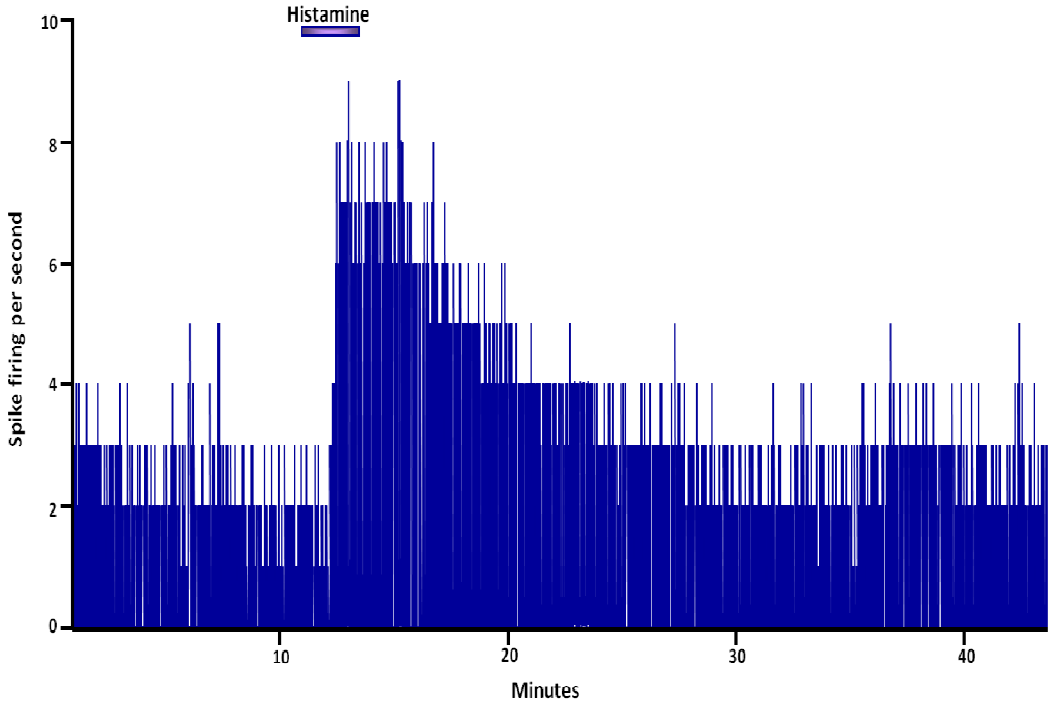


Figure 4.2.; An electrophysiology trace showing a typical rat VMN neuronal response to histamine application. The trace indicates that almost immediately after histamine is administered the spikes fired per second increase from 3 spikes per second to 7 spikes per second. There is a gradual decrease in firing rate after histamine is removed and a return to basal rate after ~ 15 minutes. The purple band indicates when histamine was applied.

Our results suggest that of the two thirds of VMN neurones that respond to histamine application, they do so with an increase in firing (97 %), illustrating that the majority of VMN neurones are excited by the transmitter histamine. The other third of VMN neurones recorded show no change in firing suggesting they are histamine unresponsive. Very few cells were found to decrease their neuronal firing rate after histamine was applied (only 4 out of 197 cells) suggesting a very small percentage of histamine neurones within the VMN are inhibited by the transmitter.

4.4.2 Histamine causes an increase in neuronal firing via the H_1R

In this study, a total of 31 VMN neurones that were responsive to the application of 5 µm histamine, were then tested by the co-application of 5 µm histamine and 50 µm pyrilamine, an H_1R antagonist. These cells showed, on average, a basal firing rate of 1.87 ± 0.93 Hz and a range of 2.76 – 0.89 Hz. Only 2 out of 31 cells (7 %) showed an increase in firing when the histamine and pyrilamine were applied together, whilst only 1 cell (2 %) decreased to below basal neuronal firing. In the majority of cells, 50 µm pyrilamine blocked the effects of histamine (28 out of 31 cells; 90 %). The two cells that increased firing rate in the presence of histamine and pyrilamine, showed an average firing rate of 3.45 ± 1.35 Hz, which is in the range of histamine alone. The one cell that decreased in neuronal firing whilst 5 µm of histamine and 50 µm pyrilamine were applied showed a neuronal firing rate of 0.51 Hz. Finally the 28 cells recorded from that were unresponsive to the histamine when pyrilamine was present, showed an average firing rate 1.28 ± 1.15. All changes in neuronal firing returned to basal levels 20 minutes after the application of the drug solution.

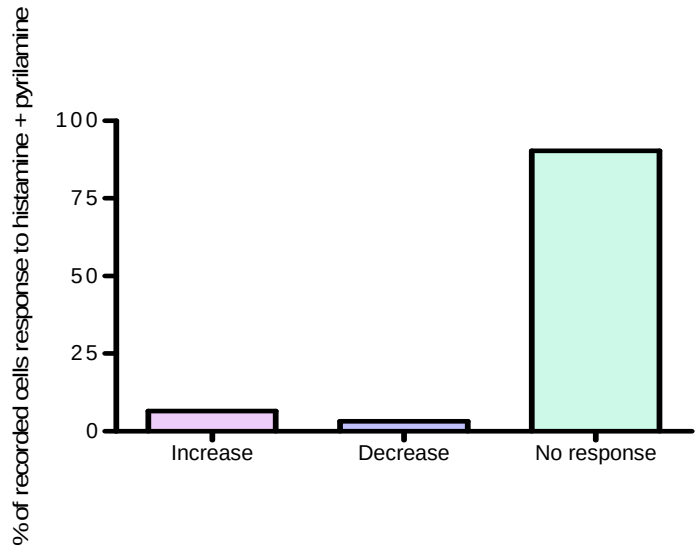


Figure 4.3.; This graph illustrates the differences in the response that cells have when a solution of histamine and pyrilamine was applied to a brain slice *in vitro*. As can be seen, the majority of cells showed no response, suggesting the H_1R antagonist pyrilamine is blocking histamine actions.

Figure 4.4 which illustrates a typical extracellular electrophysiology trace response to the application of 5 µm histamine and then the application of a solution of 5 µm histamine and 50 µm pyrilamine. As can be seen, when the histamine and pyrilamine were added together there was no change in neuronal firing (seen in over 90 % of the cells). To ensure pyrilamine was not causing long-lasting effects on the slice preparation, when possible, we added 5 µm histamine again by itself. In these cases, the neurones again responded to histamine alone.

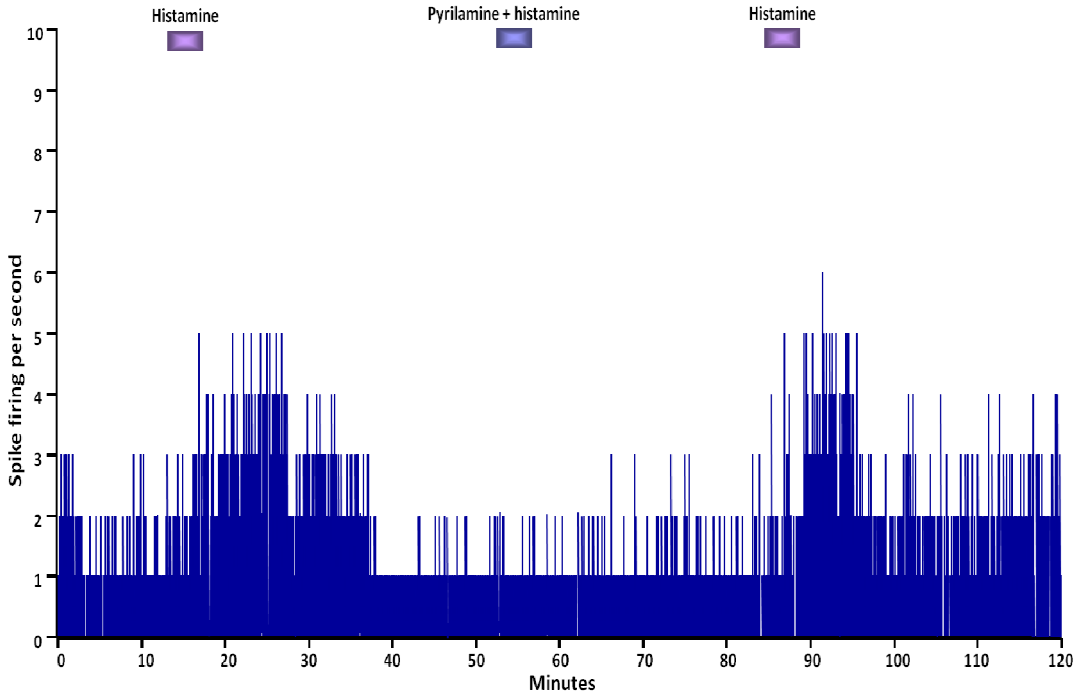


Figure 4.4.; An electrophysiology trace showing a typical rat VMN neuronal response to the application of the 50 µm pyrilamine and 5 µm histamine solution. This trace illustrates that after firstly recording a typical histamine neuronal response, when pyrilamine is added along with histamine the increase in neuronal firing is blocked. The purple band indicates when histamine was applied alone and the blue band indicates when the pyrilamine and histamine were co-administered.

4.4.3 Histamine signalling in the VMN is modified by H_3R

In this study, a total of 49 histamine-responsive VMN neurones were tested for how they reacted to the application of a solution containing 20 µm thioperamide. These cells showed on average basal firing rate of 1.29 ± 1.37 Hz and a range of 2.78 – 0.74 Hz. 43 out of 49 cells (88 %) showed an increase in firing when thioperamide was applied, whilst 1 decreased (2 %) firing and 5 had no response (10 %). Those that increased activity in response to thioperamide, showed an average firing rate of 3.15 ± 2.01 Hz. The one cell that decreased in

neuronal firing whilst thioperamide was applied showed a neuronal firing response of 0.29 Hz. Finally, the five cells recorded from that showed no changes in neuronal firing during the application of thioperamide showed an average neuronal firing rate 1.57 ± 1.18. All changes in neuronal activity were shown to return to basal levels within 20 minutes post the application of the drug solution.

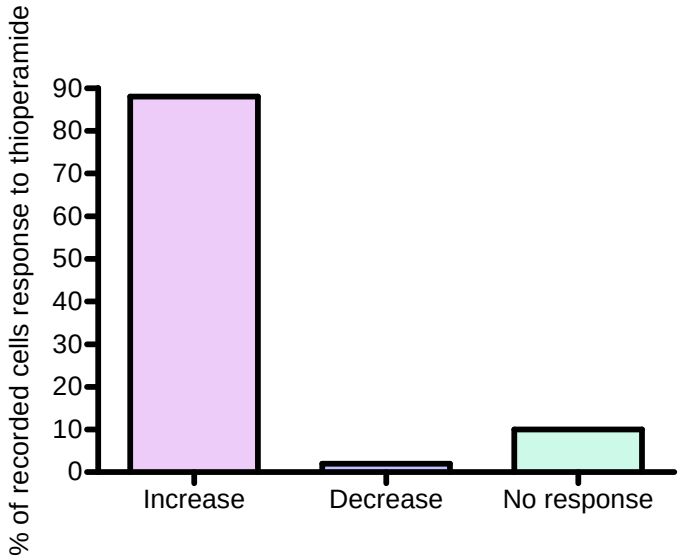


Figure 4.5.; This graph illustrates the differences in the response that cells have when the H_3R inverse agonist, thioperamide, was applied to a brain slice *in vitro*. As can be seen, the majority of cells showed a similar response as to histamine application itself, with 88 % of cells showing a increase in neuronal firing. This suggests the H_3R is present within the VMN and that their blockade can produce an excitatory increase in neuronal firing. As with histamine, very few cells showed an inhibition following the application of thioperamide (2 %) and, in the case of thioperamide, a relatively small number of cells also had no response in neuronal firing rates (10 %).

Figure 4.6 illustrates a typical trace response recorded after histamine and then thioperamide were separately applied to the brain slice. The majority of cells recorded from within the VMN illustrated that thioperamide not only caused an increase in neuronal firing, but that the response almost mimicked that to

histamine itself. The cells exposed to thioperamide showed an almost immediate increase in neuronal firing with a gradual decrease back to basal firing rates.

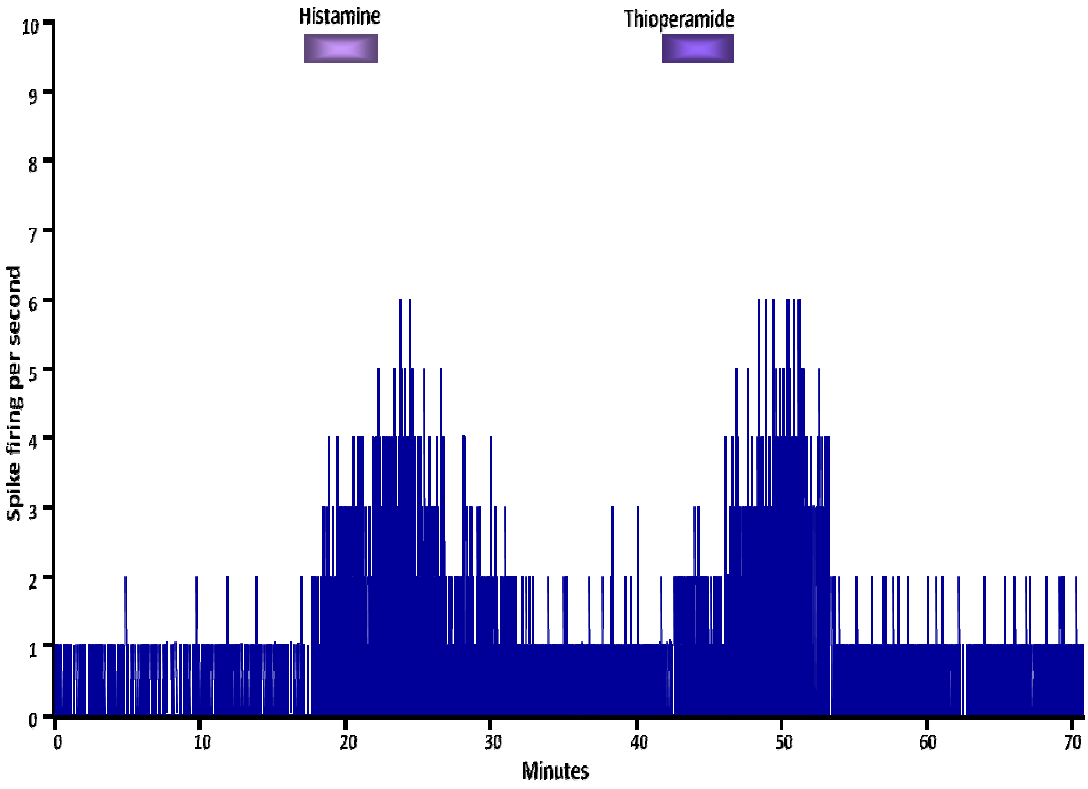


Figure 4.6.; An electrophysiology trace showing a typical rat VMN neuronal response to the application of 20 µm thioperamide. This trace illustrates that the typical histamine response is mimicked by 20 µm thioperamide. In a similar pattern to histamine, thioperamide causes a almost immediate increase in neuronal firing with a gradual return to basal levels over a 15 minute period. The purple band indicates when histamine was applied and the pink band indicates when the thioperamide was added.

Our results suggest that the H_3R is present within or near the vicinity of the VMN of the rat hypothalamus, since when the H_3R inverse agonist, thiopereamide, was applied there was an increase in firing in 88 % of cells we recorded from. Thioperamide may be causing an increase in endogenous

histamine release by stopping the negative regulation by H_3R autoreceptors. This might explain why similar responses were seen after histamine and thioperamide applications.

We have also carried out some preliminary studies to determine if the increase in firing rate that the VMN neurones were showing in response to histamine application was a presynaptic or postsynaptic event we carried out electrophysiology recordings that used low Ca^{2+} / high $MgSO_4$ aCSF (figure 4.7). Once a neurone had shown an increase in firing in response to histamine and returned to basal firing, we then place the brain slice under low Ca^{2+} / high $MgSO_4$ aCSF and applied histamine again. In the majority of neurones that received histamine diluted in low Ca^{2+} / high $MgSO_4$ aCSF there was no change in response (66 % of neurones showed no response to histamine when under a low Ca^{2+} / high $MgSO_4$ aCSF solution, n=4). During and after the application of histamine it was noted that the basal firing rate of neurone remained constant and thus suggests that application under low Ca^{2+} / high $MgSO_4$ aCSF blocks the neurones response to histamine (figure 4.7).

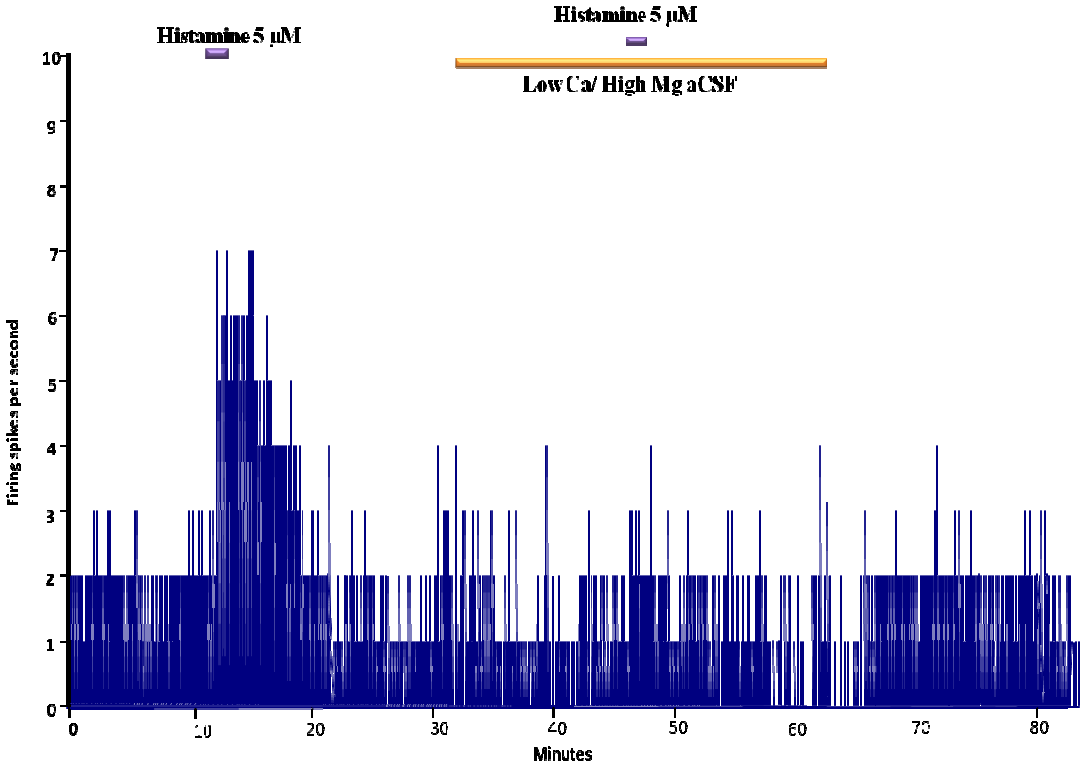


Figure 4.7.; An electrophysiology trace showing a typical rat VMN neuronal response to histamine application under low Ca^{2+}/high $MgSO_4$ aCSF. The trace indicates that almost once histamine is added under low Ca^{2+}/high $MgSO_4$ aCSF there is no increase in neuronal firing as seen when histamine is applied under the common aCSF solution. Purple band indicates where histamine was applied. Orange band indicates low Ca^{2+}/high $MgSO_4$ aCSF application.

4.4.4 Histamine activates H_3R autoreceptors within the VMN

In this study a total of 15 histamine-responsive VMN neurones were tested for their responsiveness to the application of a solution containing 20 µm of thioperamide, an H_3R inverse agonist, with or without 50 µm pyrilamine, an H_1R antagonist. These cells showed on average basal firing rate of 1.18 ± 1.29 Hz and a range of 0.89 – 2.63 Hz. All 15 cells recorded, also responded to 20 µm thioperamide with an increase in firing, however this response was lost when 50 µm pyrilamine was co-administered. The cells were shown as histamine

responsive by displaying an increase in neuronal firing when the 5 µm solution of histamine was applied to the slice. We then ensured the cell being recorded from also showed an increase in neuronal firing when thioperamide, the H_3R inverse agonist, was applied. All 15 cells showed increases in neuronal firing when either histamine or thioperamide were applied alone, but when pyrilamine was co-applied with thioperamide it was found that pyrilamine blocked the increase in neuronal firing. This suggests that the H_3R in this system is an autoreceptor as the H_1R antagonist pyrilamine is able to block the actions of the H_3R inverse agonist thioperamide. All changes in neuronal firing were shown to return to basal levels 20 minutes post the application of the drug solutions.

Figure 4.8 shows increases in neuronal firing after firstly applying 5 µm histamine and then, secondly, applying 20 µm thioperamide. Once this had been determined we then added a solution containing 20 µm thioperamide and 50 µm pyrilamine, the H_1R antagonist, and found that the excitation was lost.

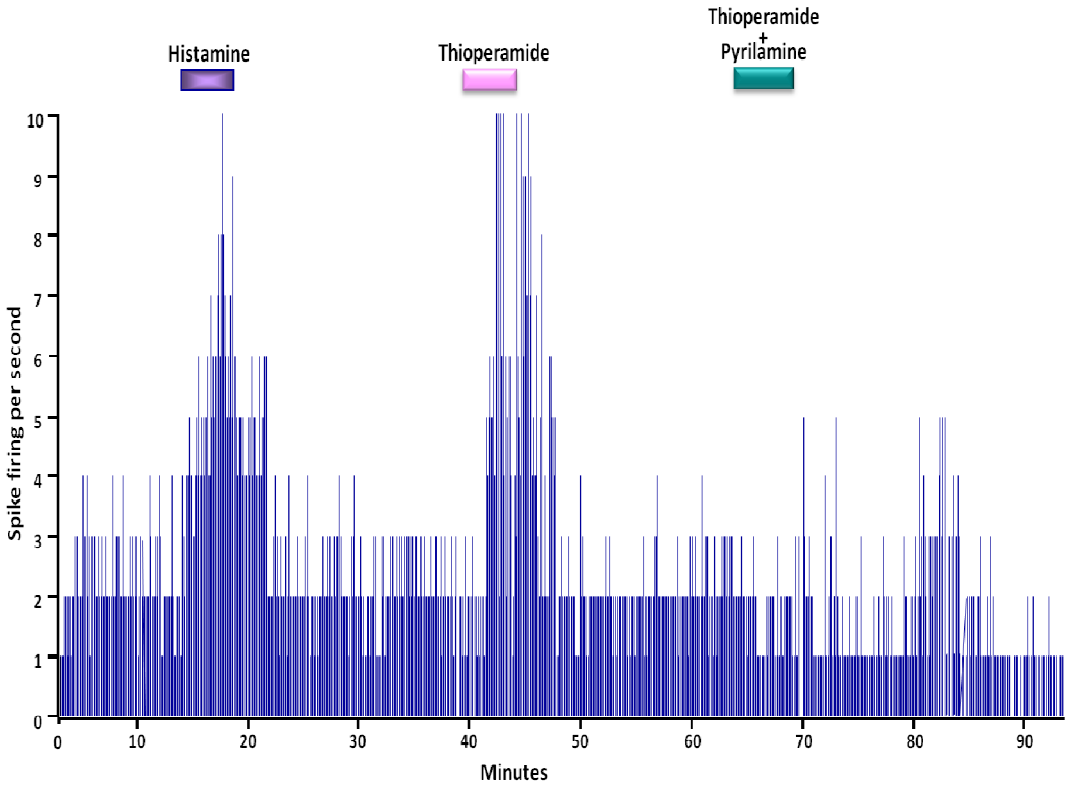


Figure 4.8.; An electrophysiology trace showing a typical rat VMN neuronal response to the application of a solution of 20 µm thioperamide plus 50 µm pyrilamine. This trace illustrates firstly a typical neuronal response when histamine is added to the slice preparation and then a typical response to the introduction of thioperamide. As can be seen, though, if pyrilamine is co-administered with thioperamide, the increase in neuronal firing was lost and neuronal firing remained at basal levels. This suggests that pyrilamine is able to block the actions of thioperamide resulting in the loss of the increase in neuronal firing. The purple band indicates when histamine was applied, the pink band indicates when the thioperamide was added and green band indicates when thioperamide and pyrilamine were co-administered.

Our results suggest we are recording the effects of thioperamide at an H_3 autoreceptor as opposed to a H_3 heteroreceptor, as when the effects of thioperamide were blocked on each occasion by the H_1R antagonist, pyrilamine. If thioperamide was acting on H_3 heteroreceptors to block the release of other transmitters, pyrilamine would have no effect on its actions. We also carried out

some recordings (results not shown) where we applied thioperamide alone after the co-treatment of thioperamide and pyrilamine to ensure that applying thioperamide alone didn't release all available endogenous histamine. This further concludes the 'no response' recording seen with the co-application of thioperamide and pyrilamine was a real result and not due to no available histamine.

4.4.5 The H_3R agonist imetit has an inhibitory effect on VMN neuronal firing

Here 22 histamine-responsive cells were recorded to determine their response to the application of 5 µm imetit. After establishing a cell was histamine responsive, we then applied imetit to establish how the cell reacts to the H_3R agonist. We found that the 22 histamine-responsive cells had an average basal firing rate of 1.86 ± 1.33 Hz and a range of 1.19 – 3.09 Hz. We found 19 (86%) of the 22 cells tested cells showed a reduction in firing rate when imetit was applied, suggesting imetit has mainly an inhibitory effect on neuronal firing within the rat VMN. The cells that responded with an inhibition in neuronal firing showed a average firing rate of 0.47 ± 0.42 Hz during and immediately post the application of the H_3R agonist. Imetit had no effect on 3 of the 22 cells (13%) we recorded, which showed an average firing rate of 1.67 ± 1.22 Hz during imetit application. No cells responded with an excitation following the application of imetit.

Figure 4.9 illustrates that after the initial excitatory histamine response is recorded and imetit is then applied, the cell then significantly reduces its neuronal firing rate almost immediately. After around 10 minutes post application of the H_3R agonist the neuronal firing rate then returns to basal levels showing this is a reversible response.

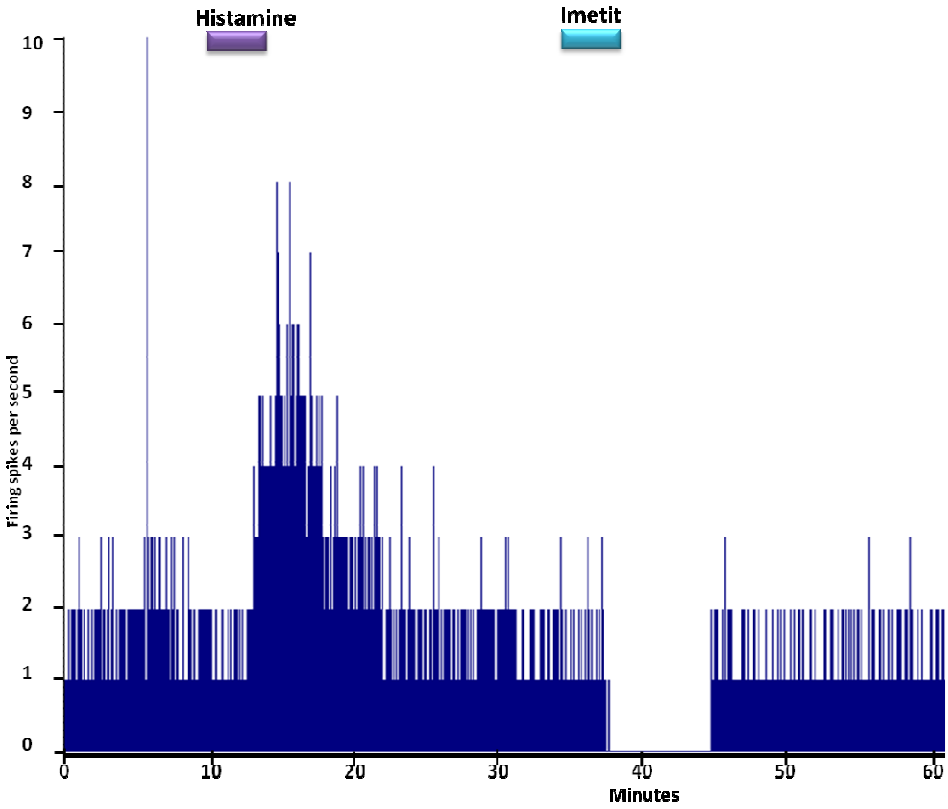


Figure 4.9.; An electrophysiology trace showing a typical rat VMN neuronal response to the application of the 20 µM imetit. This trace illustrates that after firstly recording a typical histamine neuronal response, when imetit is added the firing rate of the cell being recorded from decreases suggesting imetit has an inhibitory effect on VMN neuronal firing. Purple band indicates where histamine was applied and the green band illustrates where imetit was applied.

Our results suggest the majority of cells (86 %) within the rat VMN showed very similar response to that seen in figure 5.9, with most exhibiting an inhibitory response to imetit. An important point to illustrate is that imetit did not always completely block firing within the VMN when applied, but the majority of cells recorded from significantly reduced their firing rate. Only a small minority of cells (14 %) were unresponsive to the application of imetit and no cells showed an excitatory neuronal firing response, suggesting imetit has an inhibitory response on cells within the rat VMN.

Although all our drug applications resulted in similar responses from the VMN neurones it must be noted that the overall firing pattern we observed from the neurones we recorded from within the VMN was quite irregular with bursts of spikes occurring every minute or so.

4.5 Discussion

4.5.1 Histamine can cause an increase in neuronal firing via the H_1R within the VMN

Our results suggest that the increase in neuronal firing seen in a histamine responsive cell after the application of histamine can be blocked when the H_1R antagonist pyrilamine is co-applied. This suggests that the increase in neuronal firing seen when histamine is applied to the slice is caused by activating a H_1R within the VMN or at least within the confines of the slice. We have shown that histamine causes neuronal excitement in approximately two thirds of rat VMN neurones. The remaining cells were non responsive. It is likely that the two neurones that responded to application of histamine with a decrease in firing may have been doing so following an indirect action through other neurones within the slice, though this was not proven in the current studies. Relatively few electrophysiology studies have been carried out on the effects of histamine, but those that have been published suggest histamine can cause both the inhibition and excitation of neuronal firing rates depending on the area being investigated (Chen *et al.,* 2003; Haas *et al.,* 1977; Haas *et al.,* 1975; Haas *et al.*, 1983; Reiner *et al.,* 1987).

In some studies, histamine has been applied microelectrophoretically to the immediate environment of single cells (Haas HL., 1974; Phillis *et al.,* 1968). In most brain regions, including the cerebral cortex, brain stem and the thalamus, a

depressant effect has been demonstrated. In contrast, neurones within the hypothalamus, where the highest levels of histamine are found, an excitement in neuronal activity is often found (Haas *et al.,* 1977). In rat and cat brains, histamine caused neuronal excitement in almost 70 % of hypothalamic neurones. Chen *et al.,* (2003) also found histamine, when given at varying concentrations (1-100 µm), caused a dose-dependent increase in the firing of most hypothalamic neurones.

As mentioned previously, histaminergic neurones are found only within the TM of the posterior hypothalamus, and from here the histaminergic fibres project to most regions of the brain (Panula *et al.,* 1984; Watanabe *et al.,* 1984). The actions of histamine occur through the functioning of four types of receptors (Schwartz *et al.,* 1986). The H_1R mediates the excitation of cortical, thalamic and brainstem neurons; and are mainly responsible for the arousal actions of the histaminergic system (Diewald *et al.,* 1997). Haas *et al.,* (1975) showed the H_1R had an inhibitory action in the rat hippocampus and suggest this is probably down to an elevation of the intracellular Ca^{2+} levels. H_2 receptors have also been shown to be inhibitory in action (Haas *et al.,* 1975). However, the H_1R has also been proven to excite neurones, such as in the hippocampus (Haas *et al.,* 1983). Histamine potentiates excitation in neurons by blocking the long-lasting after hyperpolarization that follows action potentials and the accommodation of firing (Diewald *et al.,* 1997). The H_3R negatively controls the synthesis and release of transmitter (Reiner *et al.,* 1987). The H_4R is primarily expressed in hematopoietic cells indicating their function in immunomodulation (Tiligada *et al.,* 2009) and are not found within the brain.

In the results section of this chapter, we described how when histamine was applied to a rat brain slice, of the 66 % of cells that were histamine responsive, 97 % of these showed a almost immediate increase in neuronal firing rate. We have concentrated on the VMN as this is a leading feeding centre within

the rat brain, is known to contain histamine receptors and a number of research groups have suggested that when histamine is applied to a brain area within the hypothalamus an excitement in neuronal firing is recorded. As we found only four of the responding cells to show an inhibitory response to the application of histamine we too can suggest that histamine causes an excitation within the rat VMN. This response could be resulting in the animal's behavioural response to food intake after histamine is administered, suggesting histamine excites receptors within the VMN which in turn affect the appetite of the animal causing reduction in food intake.

4.5.2 Histamine signals via the H_1R causing a excitatory neuronal response within the VMN

After determining that histamine causes an excitatory response in the majority of rat VMN neurones when applied to a slice preparation, we wanted to determine if this was, as others had suggested, a H_1R-dependent response. As shown in the results section we illustrated that when pyrilamine, an H_1R antagonist was co-applied with histamine the increase in neuronal firing was blocked. This suggests that the excitatory response that histamine has on cells within the VMN is via a H_1R.

The H_1R is involved in numerous actions and is widely distributed both peripherally and centrally. Centrally, the H_1R is found post-synaptically in almost all areas of the rat brain and has been found to be involved in the regulation of appetite and feeding. Pharmacological, behavioural and knock out studies all suggest the H_1R has inhibitory effects on food intake (Fukagawa *et al.,* 1989; Sakata *et al.,* 1994; Ookuma *et al.,* 1993; Doi *et al.,* 1994; Inoue *et al.,* 1996). In particular, H_1R within the VMN are implicated in the neuronal regulation of appetite (Masaki *et al.,* 2003). However, Kow *et al.* (1989) found that histamine

effected PVN neurons with an almost exclusive excitatory action, and this excitatory action was blocked by H_1R receptor antagonists. Li *et al.* (1996) confirmed using whole-cell patch recording techniques that bath-applied histamine depolarizes membrane potential and increases firing rate in many SON neurons and that the depolarization can be blocked by H_1R antagonists and is mimicked by H_1R agonists. Electrophysiological studies have demonstrated the excitatory effect of histamine on neuronal activity in the SCN, SON and ARC is mediated by H_1R (Armstrong *et al.,* 1985, Jorgenson *et al.,* 1989, Stehl *et al.,* 1992). Zhou *et al*. (2007) used whole cell patch-clamp recording to investigate the effect of histamine on VMN neurons. They found that histamine increased the excitability of VMN neurons as indicated by membrane depolarization and increased firing rate. They suggest this effect is produced by the inhibition of potassium leakage currents through the H_1R.

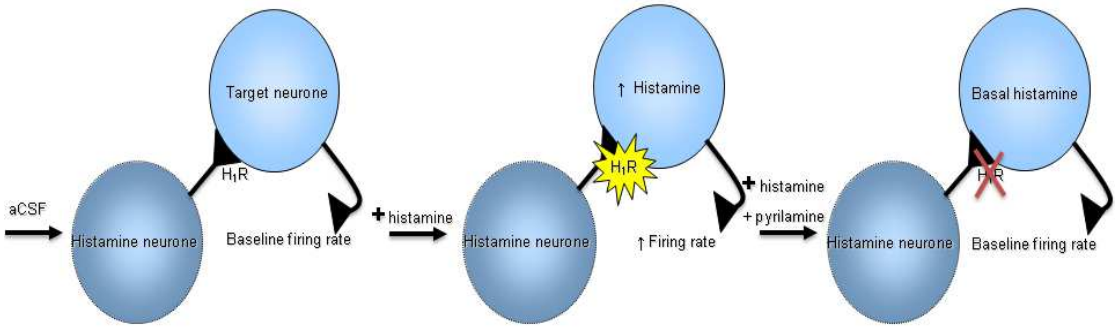


Figure 4.10.; This schematic diagram illustrates how histamine induces an excitatory response on neurones within the rat VMN via the H_1R. Histamine alone results in an increase in neuronal firing within the VMN but when the H_1R agonist pyrilamine is added this increase in neuronal firing is lost suggesting histamine is exciting cells within the VMN via a H_1R.

The majority of studies carried out that have focussed on the effects of histamine on neuronal activity within the hypothalamus have shown histamine to cause a excitation in neuronal activation as implicated by a increase in the firing rate of the neurones being recorded. These studies all found that the H_1R to be implicated in the production of this excitatory response by using selective agonists and antagonists. Thus figure 4.10, illustrates the possible pathways that histamine could be inducing its excitatory response on a VMN neurone via a H_1R.

4.5.3 Histamine can act via an H_3R to affect neuronal firing within the VMN

The H_3R has been less widely studied than the H_1R in relation to its affects on feeding behaviours, in part because it was discovered much later than the H_1R. Also the H_3R is much more complex in its physiology than the H_1R as it is a presynaptic receptor and can act as either an autoreceptor, negatively regulating the synthesis and release of histamine, or as a heteroreceptor, regulating the release of other transmitters such as serotonin and dopamine. H_3R activation inhibits the release of serotonin in the CNS, of dopamine in the CNS and the retina, and of noradrenaline in the CNS, in blood vessels and in the heart (Stark *et al.,* 1996). H_3R is found predominantly within the CNS, which makes it a good target for manipulation, avoiding the unwanted effects on peripheral systems.

H_3R mediate reduction of transmitter release (Philippu *et al.,* 1991; Schlicker *et al.,* 1994) and have been found to inhibit the firing of histaminergic neurons (Reiner *et al.,* 1987). Little research has been carried out on H_3R in the hypothalamus including the VMN and so no evidence has been gathered as to their effect in this area. We have now shown that H_3R blockade can lead to an increase in neuronal firing within the VMN of the rat brain. We have demonstrated

in the results section of this chapter that the H_3R inverse agonist thioperamide mimicked the actions of histamine when applied to a brain slice containing the VMN. Our results illustrate that not only are H_3R present within the VMN but also they can mediate an excitatory response within VMN neurones. When histamine and thioperamide are applied either centrally or peripherally a decrease in food intake is observed and an increase in neuronal activation has been demonstrated within the VMN. As the VMN is an integral player in the control of food intake we could suggest that the H_3R present in this area have roles in the regulation of appetite. Figure 4.11 illustrates how histamine can induce an excitatory response on a VMN rat neurone via the H_3R.

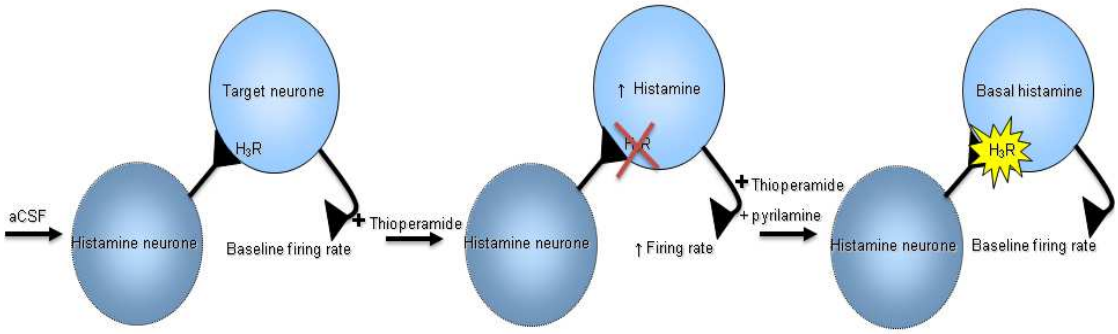


Figure 4.11.; This schematic diagram illustrates how histamine also can induce an excitatory response on neurones within the rat VMN via the H_3R. Histamine results in an increase in neuronal firing within the VMN and the H_3R inverse agonist thioperamide mimics this response also resulting in a excitatory increase in neuronal firing. This is the first evidence to date that not only suggests H_3R are present within the rat VMN but that they also cause excite neurones within this area resulting in a increase in neuronal cell firing.

4.5.4 Histamine can act via an H_3R autoreceptor to affect neuronal firing within the VMN

Our studies proved that H_3R are present within the VMN, so our next step was to determine whether these H_3R were autoreceptors or heteroreceptors. Histamine is known to have many different actions depending on the histamine receptor involved. The two receptors associated with histamine's effects on food intake are H_1R and H_3R. H_1R is located postsynaptically and H_3R is located presynaptically, and both are found within and act upon the hypothalamus. However, H_1R and H_3R have opposing effects on food intake, with the H_1R agonism decreasing and the H_3R agonism increasing food intake. By applying low Ca^{2+}/ high $MgSO_4$ aCSF, it is possible to determine whether a compound is acting postsynaptically or presynaptically. We found that of the small number of neurones tested in this way, 66% of histamine-responsive neurones did not respond to histamine when it was applied in low Ca^{2+}/ high $MgSO_4$ aCSF. If histamine is affecting the target neurone directly on postsynaptic receptors, we would expect the neurone to still respond in low Ca^{2+}/ high $MgSO_4$ aCSF. The lack of response in these neurones could suggest histamine is acting via presynaptic receptors within the VMN. Although tempting at this point to suggest the applied histamine may be acting on presynaptic H_3R, but the modulation of this receptor would be masked in low Ca^{2+}/ high $MgSO_4$ aCSF, it is equally likely with this crude experiment that the with high Ca^{2+}/ low $MgSO_4$ aCSF recordings, histamine could be acting on other neurones within the slice which then act to on the VMN neurones from which we are recording. These presynaptic neurones may themselves contain excitatory transmitters that stimulate the downstream, recorded neurone. If the presynaptic neurones contain inhibitory transmitters, then histamine may inhibit these, leading to disinhibition of the downstream, recorded neurone. In this context, it is important to note that the VMN is

surrounded by a dense network of GABAergic neurones which could potentially project into the nucleus and be affecting the response of VMN output neurones.

Thus using the co-application of the H_3R inverse agonist thioperamide and the H_1R antagonist pyrilamine we were able to establish that H_3 autoreceptors within the VMN could be exerting the excitatory response produced by VMN neurones after the application of thioperamide. We found that pyrilamine blocked the excitatory increase in neuronal firing by thioperamide, suggesting the H_3R we observed within the VMN are autoreceptors. If the H_3R were heteroreceptors, pyrilamine would have had no effect on the firing response to thioperamide in a majority of neurones.

4.5.5 H_3R agonism inhibits the excitatory response of histamine

We have also demonstrated that the H_3R agonist imetit can have an inhibitory effect on VMN neuronal firing. Our results suggest 86 % of the VMN cells showed very similar response to the application of imetit, with most exhibiting an inhibitory response by showing a significant reduction in neuronal firing whilst and immediately after the application of the H_3R agonist. These results show imetit have the opposite effects on neuronal firing within the rat VMN. As thioperamide, which is a H_3R inverse agonist, and imetit, a H_3R agonist have opposing effects on the H_3R it is not surprising they have opposite effects on the neuronal firing of VMN neurones. Thioperamide blocks the autoinhibitory actions of the H_3R and, thus, allows an increase of endogenous histamine release. Imetit activates the H_3R and, thus, encourages its autoinhibitory effects, halting the release of histamine in the slice. Consequently, thioperamide can mimic the actions of histamine whereas imetit produces opposite results. As mentioned previously, the H_3R negatively controls the synthesis and release of histamine and has been shown to inhibit the firing of histaminergic neurons

(Reiner *et al.,* 1987), which could be what we are observing in our recordings. Also, Vanni-Mercier *et al.* (2003) have reported that imetit caused a significant decrease in cell discharge and neuronal firing rate when applied to the posterior hypothalamus, but our slices did to include the TM.

It is important to note that one major drawback with extracellular electrophysiology, is that the response that we are recording could be a direct response of the drugs on that cell within the VMN or an accumulative response to a number of inputs which might themselves have histamine receptors. Due to this, it can be difficult to verify whether the response we see is a direct or indirect neuronal response to histamine and histamine receptor drugs, but the cumulative data suggests a direct action.

Chapter 5:

Proxyfan – an H_3R neutral antagonist?

5.1 Introduction

5.1.1 GPCRs and constitutive activity

G-protein coupled receptors (GPCRs) are a large family of transmembrane receptors that sense molecules outside the cell and activate intracellular signal transduction pathways resulting in cellular responses (Gbahou *et al.,* 2003). Activation of some GPCRs results in fast chemical synaptic transmission through neurotransmitter-gated ion channels. Additionally, neurotransmitters acting on GPCRs can also have slower, longer-lasting and more diverse post-synaptic actions. Transmitter action involves three steps. Firstly, the neurotransmitter molecules bind to receptor proteins embedded in the post-synaptic membrane (Fong TM., 1996). The receptor proteins then activate small protein molecules, called G-proteins (guanosine triphosphate (GTP)-binding proteins), that move freely along the intracellular face of the post-synaptic membrane. The activated G-proteins activate "effector" proteins, which can be G-protein-gated ion channels in the membrane or enzymes that synthesize second messengers that diffuse away in the cytosol and regulate ion channel function and alter cellular metabolism (Fong TM., 1996).

GPCRs are allosteric proteins that adopt inactive and active conformations in equilibrium. The active conformation can be promoted by agonists or can occur spontaneously, leading to constitutive activity of the receptor. Alternatively, inverse agonists promote an inactive conformation and decrease constitutive activity (Gbahou *et al.,* 2003). Gbahou et al (2003) suggest constitutive activity is the synthesis of a protein or enzyme at a constant rate regardless of physiological demand or the concentration of a substrate. In pharmacology, an agonist is a substance that binds to a specific receptor and triggers a response. It does so by mimicing the action of an endogenous ligand that binds to the same receptor. Agonist ligands stabilize or increase the fraction of the active state of a GPCR,

allowing it to interact with and activate a G protein. Milligan and colleagues propose that basic thermodynamics define there must be a finite probability that this active state also occurs in the absence of the agonist (Milligan *et al.,* 2003). If agonists enhance such active states, then ligands (inverse agonists) that stabilize or enrich the inactive state should be able to be identified. Therefore, an inverse agonist is able to bind to the same receptor-binding site as an agonist for the receptor, but it exerts the opposite pharmacological effect.

Heterologous expression of many GPCRs has resulted in the uncovering of ligand-independent signal transduction that increases with increasing levels of GPCR expression (Tiberi *et al.,* 1994). Many traditional 'antagonists', that block the constitutive activity of expressed GPCRs, have been reclassified as inverse agonists after establishing the constitutive activity of the GPCR is not linked to the presence of low concentrations of endogenous agonists (Milligan *et al.,* 1995). Inverse agonists are effective against only certain types of receptors, such as the histamine receptors, which have intrinsic activity without the action of a ligand upon them (Daeffler *et al.,* 2000).

Compounds that are able to bind to GPCRs without altering the balance between active and inactive states of the receptor are described as neutral antagonists and are much less common (Milligan *et al.,* 2003). An antagonist is a ligand of a receptor that blocks the ability of an agonist or inverse agonist to bind to the receptor and thus inhibits their function. Milligan *et al.* suggests that within such models, efficacy ranges from 1 (full agonism) to -1 (full inverse agonism) and neutral antagonists possess 0 efficacy (Milligan *et al.,* 1995).

Takahashi *et al.* (2003) suggest GPCRs play a major role in signal transduction and are the targets of a large number of therapeutic drugs. As mentioned before, traditional models of GPCRs require an agonist to bind to the receptor to activate the signal transduction pathways. A model used to describe agonist activation of GPCRs is referred to as the ternary complex model, which

accounts for the interactions between receptor, G-protein and agonist (De Lean *et al.,* 1980). This model has since been extended to include the observations that several GPCRs can activate G-proteins in the absence of agonists due to constitutive activity. Therefore the ternary complex model was modified to the "extended ternary complex model" or ETC (Samama *et al.,* 1993). In this model, Samama *et al.* (1993) suggest the receptor exists in an equilibrium between an inactive state (R) and an active state (R*) in the absence of a drug. Binding of an agonist to the receptors stabilizes the R* causing G-protein-coupling and activation of cellular responses. Conversely, binding of an inverse agonist stabilizes the R at the expense of R*. Takahashi *et al.* propose the primary structure and expression levels of the receptor determine the equilibrium between active and inactive receptor states and therefore determine the intrinsic basal activity of the GPCRs (Takahashi *et al.,* 2003). For wild-type receptors, R predominates, thus minimal receptor activity is present in the absence of an agonist. Alternatively, a high level of receptor expression or specific mutation in receptors can increase the concentration of R*, therefore resulting in increased activities in the absence of agonists and the enhanced susceptibility to inverse agonists. Thus the level of constitutive activity depends on both the number of spontaneously active conformations and their coupling efficiency to G proteins (Arrang *et al.,* 2007). Inverse agonists therefore abrogate constitutive activity by promoting inactive conformations of the receptor.

Protean agonists are a theoretical class of ligands that produce receptor activation but at a lower degree than that derived from spontaneous receptor constitutive activity (Kenakin T., 2001). Protean agonists are unique in that they can produce positive agonism in some GPCR systems, and inverse agonism in others, when the receptor conformation induced by an agonist shows an efficacy lower than that of the constitutively active receptor conformation (Kenakin T., 2001). For example, if the ligand produces activity in the receptor greater than

spontaneous constitutive activity, it will act like an agonist. Conversely, if the system were constitutively active and, thus, had a high number of spontaneously active sites, the ligand might reduce the activity by changing this active site to the less efficacious active site and therefore act as an inverse agonist (Kenakin T., 2001). Consequently, a protean agonist could act as either an agonist or an inverse agonist at the same GPCR, depending on the level of constitutive activity. With an none constitutively active system the ligand would be act as an agonist and in a constitutively active system it would be an inverse agonist (Arrang *et al.,* 2007. The observation of protean agonism in a system gives some evidence that the ligand being investigated produces a receptor active state of lower intrinsic efficacy than the naturally occurring constitutively active state and therefore is suggestive of selective receptor states which are a useful tool for discovery of ligand-specific receptor active-states and thus could be a useful tool in our investigations to determine the ligand-specific nature of our H_3R agonist, imetit, and H_3R inverse agonist, thioperamide.

5.1.2 Constitutive activity and the H_3R

The H_3R belongs to the class of G-protein coupled receptors which contain seven putative transmembrane domains, with an extracellular N-terminus and an intracellular C-terminus (Strakhova *et al.,* 2008). Arrang *et al.* (2007) have reviewed how the H_3R has become a GPCR of choice for studies of constitutive activity and protean agonism as H_3Rs present in the brain have been shown to exhibit high constitutive activity. Liedtke *et al.* (2003) suggest the mechanisms underlying constitutive activity might involve the carboxyl terminus of the third intracellular loop of the H_3R and is conserved among species (Lovenberg *et al.,* 1999, Hancock *et al.,* 2003). However, the functional H_3R isoforms, which all contain this sequence, show different degrees of constitutive activity. Constitutive

activity of the recombinant H_3R is, therefore, dependent on species, isoform, cell line and signaling pathway, but all of the collected data are consistent in showing the high constitutive activity of both the rat and human H_3R. Most of the compounds originally classified as H_3R antagonists are, in fact, inverse agonists, such as thioperamide, and abrogate the signaling changes generated by constitutive activity of the recombinant H_3R (Arrang *et al.,* 2007, Liedtke *et al.,* 2003).

The H_3R has been shown to signal without agonist stimulation in several recombinant receptor systems (Wieland *et al.,* 2001; Wulff *et al.,* 2002), as well as in *in vivo* systems (Morisset *et al.,* 2000), by means of constitutive activity. Pharmacological agents with high inverse efficacy would be a benefit if the clinical need is to lower basal receptor activity. Thus, inverse agonists open the possibility for a new therapeutic strategy for the H_3 receptor (Takahashi *et al.,* 2003). However, the level of H_3R constitutive activity in each specific system, such as that controlling feeding, needs to be fully determined as species differences and drug effectiveness of the H_3R agonists and inverse agonists have all been brought into question, suggesting the need for a careful evaluation of the drugs used to fully define H_3R involvement.

5.1.3 Proxyfan and its potential to act as a neutral antagonist at the H_3R

Interest in the drug proxyfan began after the discovery that the H_3R shows a high degree of constitutive activity. Several of the classical H_3R antagonists, such as thioperamide and ciproxifan, actually behave as potent inverse agonists, as they block the intracellular pathways associated with active H_3R in heterologous transfected cells (Morisset *et al.,* 2000; Rouleau *et al.,* 2002). Alternatively, the high-affinity H_3R ligand, proxyfan, acts as a protean agonist which can display the full spectrum of pharmacological activities from full agonism

to full inverse agonism (Gbahou *et al.,* 2003).

Research on proxyfan has confirmed the competition between a ligand-directed active receptor state (LR*) and a constitutively active receptor state (R*) for G proteins, predicted by Kenakin's model (Kenakin T*.,* 2001). This model suggests the intrinsic activity of proxyfan is that of an agonist when the agonist state promoted by proxyfan (LR*) has an higher efficacy than that of the constitutively active state (R*). When both states show the same efficacy, proxyfan alone will have no apparent activity on the system and therefore here proxyfan will act as a neutral antagonist (Arrang *et al.,* 2007). Alternatively, when the state promoted by proxyfan has a lower efficacy than that of constitutive activity, proxyfan behaves as an inverse agonist (Arrang *et al.* 2007).

Constitutive activity of a native GPCR can be established when its putative activation by an endogenous agonist has been excluded, which can only be achieved using a neutral antagonist (Arrang *et al.,* 2007). In the presence of constitutive activity, neutral antagonists have no effect alone, but will block the effects of agonists and inverse agonists. Constitutive activity at H_3 autoreceptors inhibits histamine release (Morriset *et al.,* 2000). By suppressing H_3R constitutive activity, inverse agonists such as thioperamide enhance histamine release, whereas agonists such as imetit inhibit its release. Here, proxyfan alone would have no effect on histamine release, but will blocks the effects of both thioperamide and imetit, and therefore act as a neutral antagonist (Clark *et al.,* 1996). The lack of effect of proxyfan alone illustrates antagonism of endogenous histamine does not contribute to the histamine-releasing effect of thioperamide or other drugs (Clark *et al.,* 1996, Meier *et al.,* 2004). Fox and colleagues demonstrated that H_3 autoreceptors that inhibit histamine synthesis in brain also show constitutive activity (Fox *et al.,* 2002). Histamine released from histamine neurons is metabolized to tele-methylhistamine (t-MeHA). Levels of t-MeHA are, thus, a reliable indication of histamine neuron activity (Schwartz *et al.,* 1991). H_3R

inverse agonists enhance t-MeHA levels by abolishing the constitutive block caused by H_3 autoreceptors (Clark *et al.,* 1996). Proxyfan acts as a neutral antagonist by antagonizing the increase in t-MeHA levels induced by thioperamide or ciproxifan and the decrease induced by imetit (Clark *et al.,* 1996; Meier *et al.,* 2004). Proxyfan also has been shown to act as a neutral antagonist in H_3R-mediated intake of water. Here Fox *et al.* (2002) showed that proxyfan attenuates both agonist and inverse agonist effects on drinking behaviour in mice.

One issue with proxyfan is that it appears to be very species and organ dependent in its actions and ability to act as a protean agonist. Where inverse agonists, such as ciproxifan, induce arousal in all species (Ligneau et al. 1998), the effect of proxyfan on the sleep–wake cycle depends on the species. In the mouse, proxyfan increases wakefulness, decreases sleep, and therefore behaves as a full inverse agonist (Morisset *et al.,* 2000). Conversely, in the cat, proxyfan increases sleep and shows the same effect as the H_3R agonist (R)-α-methylhistamine (Morisset et al. 2000). In the rat, proxyfan acts as an inverse agonist on histamine neuron activity (Morisset *et al.,* 2000), but as a full agonist on fear memory (Baldi *et al.,* 2005). Here, Baldi *et al.* (2005) reported that rats that received systemic administrations of proxyfan showed a stronger response to the footshock-context association. Proxyfan can mimic the memory-enhancing effect of H_3R agonists administered in the amygdala (Cangioli *et al.,* 2002) or in the hippocampus (Giovannini *et al.,* 2003), in contextual fear conditioning. Full agonism of proxyfan has also been observed in the cat, where it significantly increases deep slow-wave sleep, without affecting wakefulness, whereas in the mouse proxyfan behaves as a full inverse agonist (Gbahou *et al.,* 2003), mimicking the arousal effect of ciproxifan (Parmentier *et al.,* 2002). Proxyfan has also acted as a partial agonist in a murine dipsogenic model (Fox *et al.,* 2002), as mentioned above. Gbahou *et al.* (2003) suggest competition between the active and inactive forms of the H_3R receptor may be responsible for the variety of

pharmacological responses observed in different brain regions and in different species. The pharmacology of H_3R is very complex, and Passani and colleagues suggest that receptor polymorphism with differential splice variant distribution in the CNS, potential coupling to different G protein signaling pathways and heterogeneity contributes to the numerous effects of histaminergic ligands when tested in different brain regions and across animal species allowing a range of behavioural responses to be observed (Passani *et al.,* 2004). Therefore, protean agonism indicates that a single drug might produce different responses, depending on the constitutive activity of the system.

Morriset *et al.* (2000) suggest the pharmacological profile of proxyfan depends on the system being tested. Thus it depends on the equilibrium between the active and inactive conformations of the receptor and/or the stoichiometric ratio of the receptor to the various G proteins. Thus, Gbahou *et al.* 2003, suggest that a single drug such as proxyfan may belong to all of the classical classes of ligands (full agonists, partial agonists, neutral antagonists, partial inverse agonists, and full inverse agonists). Therefore, proxyfan could be used as a tool to discover ligand-specific receptor active-states and, thus, could be used in our investigations to determine the ligand-specific nature of our H_3R agonist, imetit, and H_3R inverse agonist, thioperamide.

5.2 Aims and Objectives

5.2.1 Does proxyfan alone affect feeding in Sprague Dawley rats?

Proxyfan has been shown by a number of research groups to act in differently in various models, including as a H_3R agonist, inverse agonist, protean agonist and neutral antagonist (Clark *et al.,* 1996, Morisset *et al.,* 2000, Gbahou *et al.,* 2003, Baldi *et al.,* 2004, Passani *et al.,* 2004, Meier *et al.,* 2004). Fox *et al.*

(2002) published a paper illustrating how proxyfan can block the actions on drinking behaviour of ciproxifan, a H_3R inverse agonist, and to attenuate R-α-MeHA effects on water intake. As proxyfan has not been investigated into its effects on food intake and appetite behaviour, we wanted to determine whether alone it would act as a H_3R agonist, inverse agonist, a protean agonist or a neutral antagonist.

Levels of endogenous histamine in the whole brain may vary greatly across the time of day, and also in regions of the brain specifically involved in appetite regulation. Thus, we measured the effects on normal food intake of proxyfan at both lights out in animals that were fed *ad libitum* and at lights on in overnight fasted animals, ensuring a spectrum of endogenous histamine concentrations would be tested.

5.2.2 *Can proxyfan attenuate the actions on food intake of other H_3R drugs?*

Once we had established whether proxyfan had any effect on food intake alone, we then wanted to determine whether proxyfan could act as a neutral antagonist at the H_3R and attenuate the actions on food intake of both the H_3R agonist, imetit, and the H_3R inverse agonist, thioperamide, when co-administered. Proxyfan has been seen to act in a number of ways, to attenuate the actions of some H_3R drugs and not others (Fox *et al.,* 2002), and have effects on some behaviours in certain species and not in others (Morisset *et al.,* 2000, Gbahou *et al.,* 2003). Therefore, we aimed to determine whether proxyfan could indeed attenuate or block the effects that imetit and thioperamide have on food intake in male Sprague Dawley rats.

5.2.3 Can proxyfan act as a neutral antagonist on feeding-related neurones in vitro?

Once we determined if proxyfan affects food intake when administered alone or when co-administered with H_3R drugs, we aimed to determine the effects proxyfan has at a neuronal level in an *in vitro* slice preparation using single-unit extracellular electrophysiology. Here we plan to determine the effects proxyfan has on neurons within the VMN when applied via a perifusion line. We then aim to determine whether, when co-applied, proxyfan can block or attenuate the actions of imetit or thioperamide on neuronal firing rates of neurons within the VMN.

5.3 Methods

5.3.1 Animals

For the feeding studies, male Sprague-Dawley rats (225 - 275 g, Charles River, UK) were used and for the electrophysiology studies male Sprague-Dawley rats, aged 6 - 8 weeks (Charles River, UK) were used. All animals were adapted to a 12 h/ 12 h light-dark cycle (lights on 08:00 - 20:00), at least one week prior to the experiments starting. Rats were housed in a temperature-controlled room (~ 22 ± 1 ^{o}C) with relative air humidity 40 - 60 %. Rats had free access to food (Beekay, UK) and water unless otherwise stated. Animals used in the electrophysiology studies were culled using a UK Home Office recommended Schedule 1 procedure. These and all other experiments conformed with the Home Office (Animals Procedures Act of 1986 and local ethical review.

5.3.2 Compounds

Compounds used in the behavioural studies were administered via injection into the intraperitoneal cavity (i.p.) of the rat. Imetit and thioperamide (both Sigma) were diluted with isotonic saline to allow injection of 10 mg/kg and 2 mg/kg of body weight, respectively. Proxyfan (Tocris) was made up to 5, 2, 0.5 and 0.2 mg/kg body weight for the feeding experiments and to 5 mg/kg body weight when co-administered with either imetit or thioperamide. All drugs were diluted to their required concentrations immediately prior to use.

For the extracellular electrophysiology procedures compounds were used at the following concentrations: histamine 5 µM; thioperamide 20 µM; imetit 5 µM; proxyfan 20 µM. For all compounds an aliquot was thawed, diluted to the appropriate concentrations in fresh aCSF (see previous chapter for details on how to make aCSF solution) and bath applied to the brain slice being recorded from via a perifusion line immediately before use.

All concentrations were determined following a review of the literature (Fox *et al.* 2002).

5.3.3 Proxyfan and its effects on food intake

Rats were caged singly 24 hours before i.p. injections we administered. Drug-treated rats were given 5 mg/kg of body weight of proxyfan in a volume of 1ml/kg of body weight and control rats received a volume of 1 ml/kg of body weight of saline, i.p. Satiated rats given the i.p. injection of proxyfan at lights on (08:00 h) were allowed free access to food until they were injected with proxyfan. Rats receiving an i.p. injection of proxyfan at lights off (20:00 h), had their food removed 2 hours before lights out to ensure no pre-feeding was carried out before the experiment. Pre-weighed food was returned to the rat 2 minutes after

receiving the injections and food consumption was then measured at 1, 2 and 4 hours post injection.

5.3.4 Does proxyfan act as a neutral antagonist in the feeding model?

Again, rats were caged individually 24 hours before any injections or experiments were carried out. All injections were i.p. and in a volume of 1 ml/kg body weight. Rats being tested for their food behaviour response to imetit received an injection of 10 mg/kg body weight of imetit at lights off (20:00 h) 15 minutes after receiving an injection of 5 mg/kg body weight of proxyfan. Rats being tested for their feeding response to thioperamide received an injection of 2 mg/kg body weight of thioperamide 15 minutes after receiving an injection of 5 mg/kg body weight of proxyfan, again at lights off (20:00). Pre-weighed food was returned to the rat 2 minutes after receiving both injections and food consumption was then measured at 1, 2 and 4 hours post injection.

5.3.5 The effects of proxyfan on neurones within the VMN

An account of how our extracellular rig is set up and how our slices are prepared and recorded from is detailed in the previous chapter.

Once a neurone was identified and a stable baseline firing rate was established, 5 μM histamine was applied by perifusion for 4 minutes. If an excitatory histamine response was recorded, histamine was washed off during 20 minute application of normal aCSF. Proxyfan was then applied via the perfusion line for 4 minutes and the cells response was recorded. Normal aCSF was then applied for 20 minutes to again allow the cell's firing to return to baseline levels. Thioperamide was then co-applied with proxyfan for 4 minutes to determine whether proxyfan would block the excitatory effects that thioperamide has been

shown to produce (see previous chapter). Again, normal aCSF was the applied for 20 minutes to wash off the effects of the drugs and baseline levels of firing were returned. Thioperamide was then applied alone for 4 minutes to check the effects when applied alone. Finally normal aCSF was again administered for 20 minutes to allow neuronal firing to return to baseline levels.

For the imetit electrophysiology study, after an excitatory response to histamine was recorded and neuronal firing was allowed to return back to basal levels after 20 minutes of normal aCSF application, imetit was appled for 4 minutes and the response recorded. Normal aCSF was then applied to allow the drug to wash off and neuronal firing to return to normal levels. A solution of imetit and proxyfan was then applied over a 4 minute period and the response was recorded to determine whether proxyfan could block the actions that imetit alone. Finally, normal aCSF was again administered for 20 minutes to allow neuronal firing to return to baseline levels.

5.3.6 Statistical analysis

All data are presented as mean ± SEM. Data from feeding groups were analysed using a two-way analysis of variance (ANOVA) with repeated measures. Significance was taken at $P < 0.05$.

Recordings were only taken from the neurones in the dorsomedial region of the VMN. A VMN neurone was considered to have responded if the firing rate increased or decreased by 20 % relative to the firing rate 5 minutes prior to application of proxyfan, imetit, thioperamide or a combination of these drugs. Responses are given as a mean firing rate ± S.E.M. and show significance at $P < 0.05$ using an unpaired t-test.

5.4 Results

5.4.1 Proxyfan alone has no effect on food intake in rats

Firstly, we determined whether different doses of the H_3R drug proxyfan affect the feeding behaviour of Sprague-Dawley rats that had free access to food at the beginning of the light phase. We found that an injection of proxyfan at 0.2, 0.5, 2 and 5 mg/kg body weight had no significant effects on food consumed at 1, 2 or 4 hours post injection (figure 5.1). Rats that received the saline injection cumulatively had eaten 0.12 ± 0.08 g at 4 post injection, which is very similar to the rats that received either 0.2, 0.5, 2 or 5 mg/kg body weight of proxyfan (0.65 ± 0.8 g, 0.75 ± 0.9 g, 0.1 ± 0.1 g and 0.13 ± 0.07, respectively). These results suggest both high and low doses of proxyfan have little effect on the food intake of satiated rats.

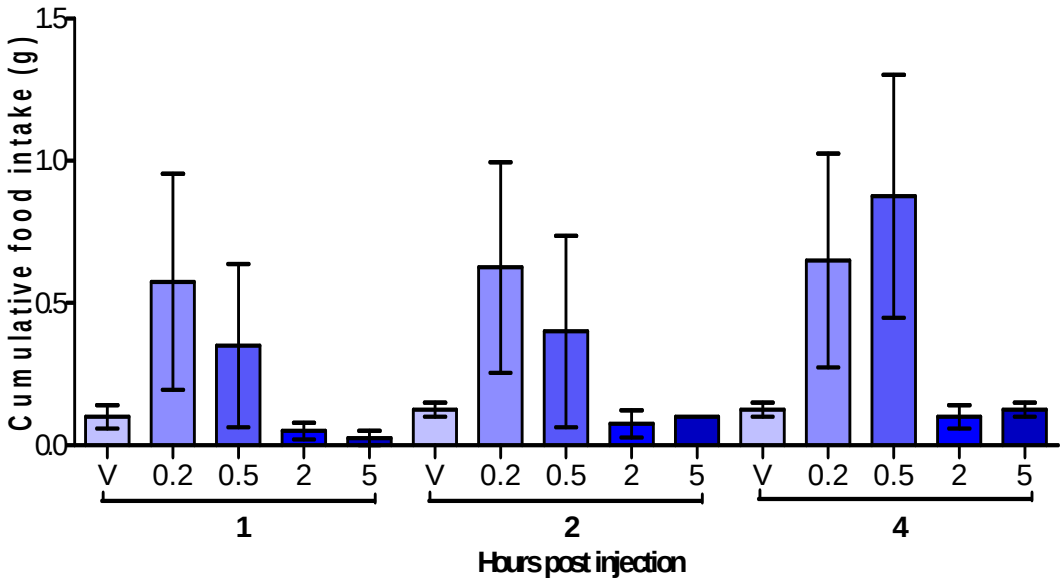


Figure 5.1.; Cumulative food consumption measured at 1, 2 and 4 hours after i.p. injection with proxyfan (n = 4) or saline (n = 4) during the day time (08:00 h). Food intake over this period is very low in these nocturnal animals. Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferronni *post hoc* test was carried out. Significance = n/s.

We then determined whether these same doses of proxyfan (0.2, 0.5, 2 and 5 mg/kg body weight) had any effects on food intake of rats when fed at normal night time feeding (20:00 h). We found that an injection of proxyfan at any of the concentrations tested had no effect on food intake, with all animals within the drug groups eating similar amounts to the control group (figure 5.2). Rats that received an injection of saline ate on average 9.4 ± 1.8 g at 4 h post injection, whereas rats receiving an injection of 0.2, 0.5, 2 and 5 mg/kg body weight of proxyfan ate 9.9 ± 1.3 g, 9.0 ± 0.6 g, 9.6 ± 0.5 g and 8.8 ± 1.1 g of food, respectively.

Figure 5.2.; Cumulative food consumption measured at 1, 2 and 4 hours after i.p. injection with proxyfan (n = 4) or saline (n = 4) given at lights out (20:00 h). Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferronni *post hoc* test was carried out. Significance = n/s.

5.4.2 Proxyfan blocks imetit-induced hyperphagia in rats

As none of our concentrations of proxyfan had shown any affects on rat food intake alone, we decided to use the highest concentration of proxyfan tested (5 mg/kg body weight), which is similar also to concentrations used by other research groups. Our research showed that when an injection of imetit was given after an injection of proxyfan, no increase in food intake was noted from control levels whereas when an injection of imetit was administered after an initial injection of saline a significant increase in food intake was observed 1 hour post the injection of imetit. This suggests proxyfan is blocking the effects imetit has on rodent feeding. We found rats that received a double saline injection ate on average 3.89 ± 0.72 g of food 1 hour post the first injection. Rats that received a proxyfan injection and then an injection of saline 15 minutes later ate on average 3.37 ± 1.11 g of food 1 hour post the first injection, rats that received a injection of saline and then an imetit injection ate on average 4.89 ± 0.56 g of food and rats that received an injection of proxyfan and then an imetit injection 15 minutes later at on average 3.58 ± 0.67 g of food. Only rats that received the injection of saline and then later were administered a second injection containing imetit were found to eat significantly more ($P < 0.05$) than the control group and the proxyfan-vehicle or proxyfan-imetit group. Significance was lost by two hours after the first injection.

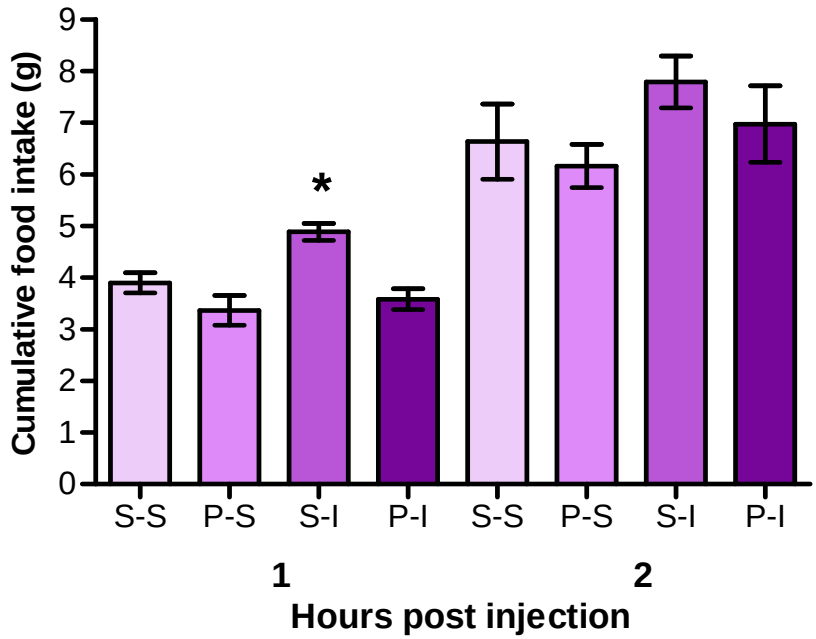


Figure 5.3.; Cumulative food consumption measured at 1 and 2 hours after i.p. injections with; saline (S) or proxyfan (P) followed by saline (S) or imetit (I) (n = 6). There are four different drug groups tested and rats received two injections 15 minutes apart. These groups include; saline-saline, proxyfan-saline, saline-imetit and proxyfan-imetit. Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferronni *post hoc* test was carried out. $P < 0.05$.

5.4.3 Proxyfan blocks thioperamide-induced hypophagia in rats

We found that when an injection of thioperamide is administered prior to an injection of saline rats consumed significantly less food than when thioperamide is injected after receiving an injection of proxyfan suggesting proxyfan is able to block the anorexic effects of thioperamide. We found that when we administered a double injection of saline rats ate on average 1 hour post the first injection 3.89 ± 0.72 g of food. Rats that were given a proxyfan injection and then administered with an injection of saline 15 minutes later, ate on average 3.37 ± 1.11 g of food,

rats that received an injection of saline and then thioperamide ate on average 2.56 ± 0.51 g of food and rats that were administered with proxyfan and then thioperamide ate on average 3.73 ± 1.69 g of food. Only rats that received an injection of saline prior to receiving an injection of thioperamide were found to eat significantly less ($P < 0.05$) food than animals receiving a double saline injection or even those receiving proxyfan-saline or proxyfan-thioperamide. These results suggest proxyfan is blocking the inhibitory effect that thioperamide has on rodent feeding. Significance was lost by 2 hours post the first injection.

Figure 5.4.; Cumulative food consumption measured at 1 and 2 hours after i.p. injections with; saline (S), proxyfan (P) or thioperamide (T) (n = 6). There are four different drug groups tested and rats received two injections 15 minutes apart. These groups include; saline-saline, proxyfan-saline, saline-thioperamide and proxyfan-thioperamide. Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferronni *post hoc* test was carried out. $P < 0.05$.

5.4.4 Proxyfan alone has no effect on VMN neuronal firing

In this experiment, at total of 20 spontaneously firing histamine-responsive VMN neurons were tested for their responsiveness to the application of 20 μM proxyfan. We firstly recorded an excitatory response to histamine from the 20 cells tested before we then applied proxyfan to the bath surrounding the brain slice and determined 100 % of the cells we recorded from had no change in neuronal firing rate. These cells showed an average basal firing rate of 1.35 ± 0.98 Hz and a range of 2.61-1.02 Hz. In our study, none of the 20 cells showed a change from basal firing rate when proxyfan was applied over a four minute period to the brain slice. The average firing rate during the administration of the drug was 1.24 ± 0.84 Hz which was not significantly different from basal firing levels. Figure 6.5 shows that after a basal rate of VMN neuronal firing was established over a ten minute period, histamine was then added and there was a rapid increase in neuronal firing which gradually returned to basal levels after around 15 minutes after the application of the drug. As can be seen in an example of a recording (figure 5.5), when proxyfan is added after the effects of histamine have been washed off, there was no change in firing rates from basal level.

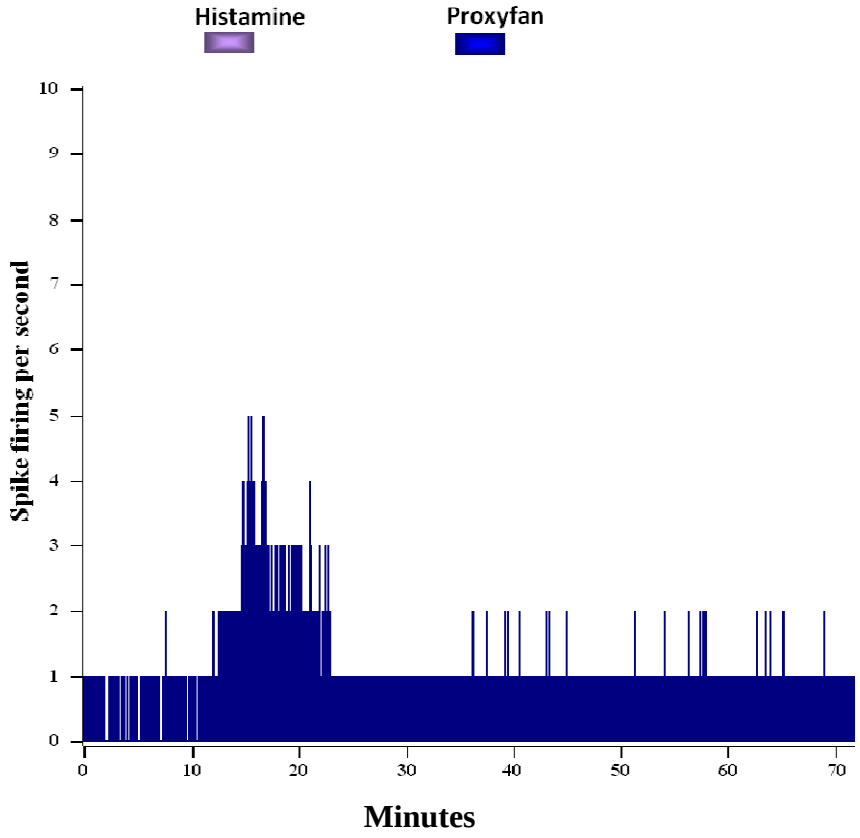


Figure 5.5.; An electrophysiology trace showing a typical rat VMN neuronal response to the application of the 20 µM proxyfan. This trace illustrates that after firstly recording a typical histamine neuronal response when proxyfan is added the firing rate of the cell being recorded from does not change from basal levels suggesting proxyfan when administered alone has no effect on VMN neuronal firing. Purple band indicates where histamine was applied and light blue band indicates where proxyfan was added.

5.4.5 Proxyfan can block the inhibition in neuronal firing caused by imetit

After determining that proxyfan had no effect on neuronal firing when applied alone to a brain slice, we then wanted to establish whether proxyfan could block the inhibitory effects of the H_3R agonist imetit on these cells. After establishing recording from a firing cell that was histamine responsive, we then applied imetit alone to establish how the cell reacts to the H_3R agonist. If the cell was both

histamine and imetit responsive, we then co-applied proxyfan with imetit. We recorded from 22 histamine-responsive cells with an average basal firing rate of 1.86 ± 1.33 Hz and a range of 1.19-3.09 Hz. We found 19 (86 %) of the 22 cells tested cells showed a reduction in firing rate when imetit was applied, suggesting imetit has mainly an inhibitory effect on neuronal firing within the rat VMN. The cells that responded with an inhibition in neuronal firing showed an average firing rate of 0.47 ± 0.42 Hz during and immediately after the four minute application of the H_3R agonist. Imetit had no effect on 3 of the 22 cells (14 %) we recorded from with an average firing rate of 1.67 ± 1.22 Hz. No cells responded with an excitation of neuronal firing to the application of imetit.

After we established imetit causes a reduction in neuronal firing, we then wanted to determine whether proxyfan could block this effect. Here we co-applied proxyfan with imetit for 4 minutes onto 20 histamine-responsive cells and found that, in all cells recorded, proxyfan blocked the inhibitory actions of imetit. All 20 cells showed no change from basal firing levels when proxyfan and imetit are co-applied suggesting proxyfan is able to block the inhibition in neuronal firing rates seen when imetit alone is added.

Figure 5.6 illustrates that after the initial excitatory histamine response is recorded and imetit is then applied the cell then significantly reduces its neuronal firing rate almost immediately. After around 10 minutes post application of the H_3R agonist the neuronal firing rate then returns to basal levels showing this is a reversible response. It is then seen that once the response to imetit is thoroughly washed off (after a 20 minute period), an application of proxyfan and imetit was added and no change from basal levels of neuronal fifing were recorded. We continued to record for approximately 20 minutes post the application of proxyfan and imetit in case of a late response but in no cases was there any deviation from basal firing rates.

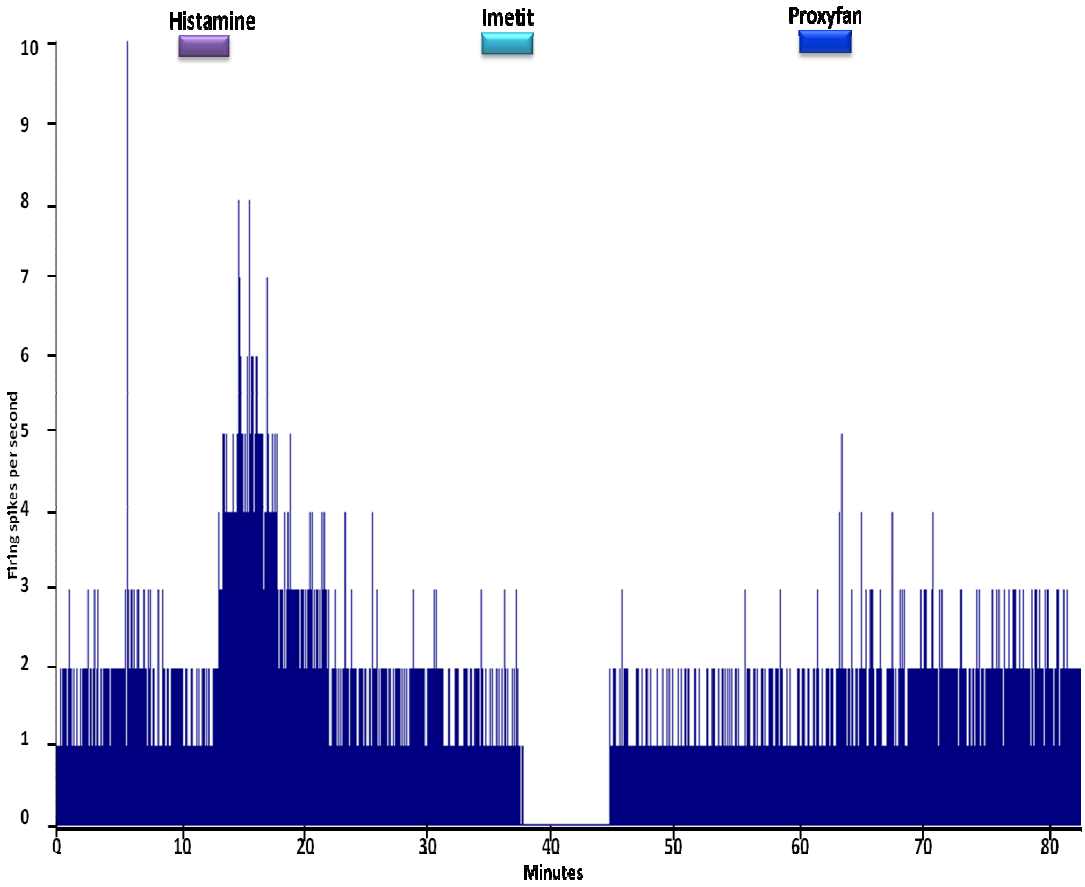


Figure 5.6.; An electrophysiology trace showing a typical rat VMN neuronal response to the application of the 20 µM imetit and then the application of 20 µM imetit with 20 µM proxyfan. This trace illustrates that after firstly recording a typical histamine neuronal response, when imetit is added the firing rate of the cell being recorded from decreases suggesting imetit has an inhibitory effect on VMN neuronal firing. When proxyfan is then co-administered with imetit there is no change in neuronal firing rates from basal levels suggesting proxyfan is able to block the actions of imetit and act as a neutral antagonist. Purple band indicates where histamine was applied, the green band illustrates where imetit was applied and the blue band indicates when either the imetit and proxyfan solution was added.

Our results suggest the majority of cells (approximately 86 %) within the rat VMN showed very similar response to that illustrated in figure 4.9 in the previous chapter, with most having a inhibitory response to imetit by showing a significant

reduction in neuronal firing whilst and immediately after the application of the H_3R agonist. Only a small minority of the cells recorded (14 %) were unresponsive to the application of imetit and no cells showed an excitatory neuronal firing response suggesting imetit has an inhibitory response on cells within the rat VMN. All cells that we co-applied proxyfan and imetit had no changes in neuronal firing rate suggesting proxyfan is blocking the effects of imetit in our system and is, thus, acting as a neutral antagonist.

5.4.6 Proxyfan can block the excitation in neuronal firing caused by thioperamide

In this study, we recorded from 16 spontaneously firing cells within the rat VMN to study their response to neuronal firing rate when proxyfan is co-applied with the H_3R agonist thioperamide. We first established that the cell we were recording from was histamine responsive and we then determined that proxyfan had no effect when applied alone to the cell (results as above). Once these recordings were established we then co-applied proxyfan with thioperamide and found this had no effect on the neuronal firing rate of the cells. All 16 cells showed an increase in neuronal firing when histamine was added, no change in neuronal response when proxyfan was applied alone, and again no change from basal firing rate when proxyfan and thioperamide were co-applied. To ensure these cells could still respond to thioperamide alone, we then applied thioperamide without proxyfan to the brain slice. We found the cells then showed an excitatory response with an almost immediate increase in neuronal firing rate. This suggests proxyfan is able to block the excitatory effects of thioperamide without any long-term effects. On average, cells showed a basal firing rate of 1.35 ± 0.57 HZ with a range of 2.61-1.02 Hz. The average firing rate of the cells during and immediately

post the application of the proxyfan and thioperamide solution was 1.33 ± 0.93 Hz. Confirming what we saw in the previous chapter, when thioperamide was applied alone the average firing rate was 3.15 ± 2.01 Hz during and immediately post the drugs application.

Figure 5.7 illustrates a typical extracellular electrophysiology trace response to the co-application of proxyfan and thioperamide. The typical increase in neuronal firing after histamine application is seen first, then a return to basal firing levels, before proxyfan is then applied. After these first two responses have been established we then added the proxyfan and thioperamide solution and as is shown in figure 6.7, and no change from neuronal firing was recorded. To ensure the cell was still thioperamide responsive, we then added thioperamide alone. An increase in neuronal firing was produced showing that proxyfan was blocking the effect of thioperamide, and that it was not that the cell was unresponsive to thioperamide.

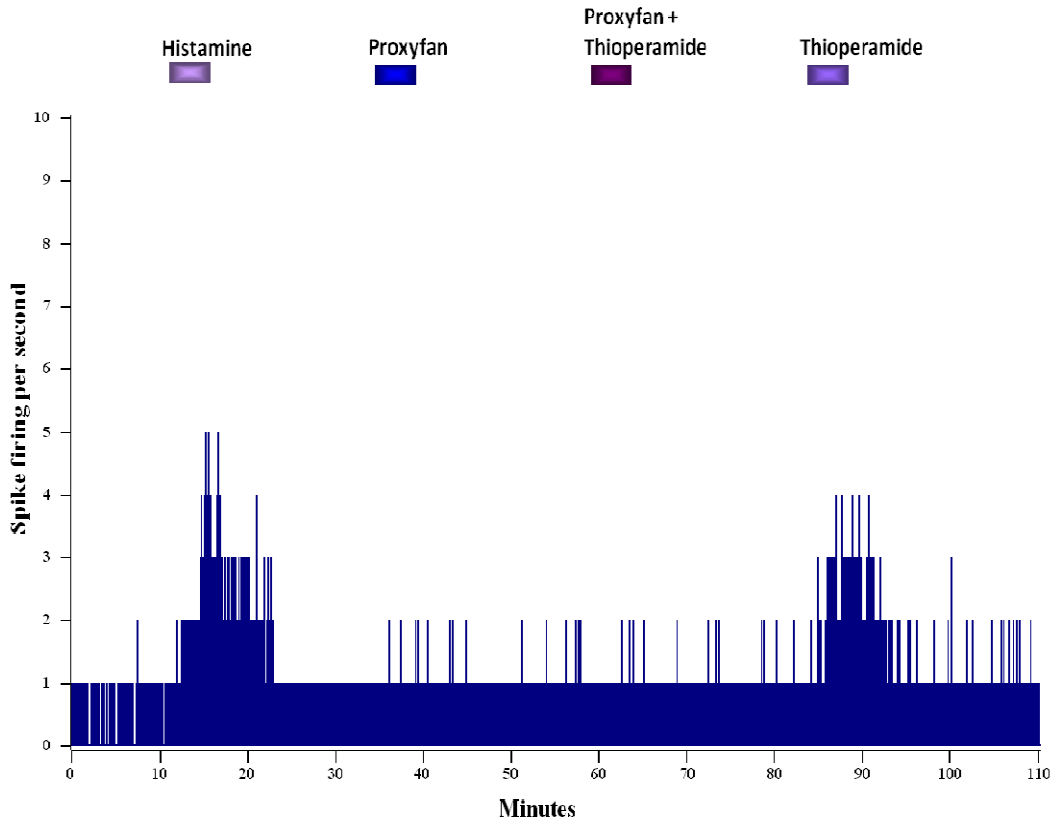


Figure 5.7.; An electrophysiology trace showing a typical rat VMN neuronal response to the application of the 20 µM proxyfan and then the application of 20 µM thioperamide with 20 µM proxyfan. Finally, 20 µM thioperamide was applied alone. This trace illustrates that, after firstly recording a typical histamine neuronal response, when proxyfan is added, the cell shows no response and the firing rate remains at basal levels. When proxyfan is co-administered with thioperamide, again the cell shows no deviation in its firing rate. When thioperamide alone is added, an excitatory response is noted with the firing rate rapidly increasing. These results suggest proxyfan is able to block the actions of thioperamide and act as a neutral antagonist. Purple band indicates where histamine was applied, the light blue band illustrates where proxyfan was applied, the pink band indicates where the solution of proxyfan and thioperamide was added and the red band indicates where thioperamide alone was added.

As all the cells showed a excitatory response to both histamine and thioperamide yet showed no change in response when proxyfan plus thioperamide was applied, our results suggest proxyfan is blocking the excitation in neuronal firing produced by the H_3R inverse agonist and thus is acting as a neutral antagonist in our system.

Although all our drug applications resulted in similar responses from the VMN neurones it must be noted that the overall firing pattern we observed from the neurones we recorded from within the VMN was quite irregular with bursts of spikes occurring every minute or so.

5.5 Discussion

5.5.1 Proxyfan acts as a neutral antagonist at the H_3R at a behavioural and neuronal level

Although proxyfan has been shown to have little effect alone in some systems (Clark *et al.,* 1996, Meier *et al.,* 2004) and to alter others significantly (Baldi *et al.,* 2004, Gbahou *et al.,* 2003), the effect of this drug has never been tested for its effects on feeding in the rat and, to our knowledge, the effects of proxyfan on the electrical activity of non-histaminergic cells have not been published.

As we have illustrated above, the H_3R compound has no effect on food intake when administered systemically in both satiated and non-satiated rats. This shows that when endogenous brain histamine levels are both high (non-satiated animals) and low (in satiated animals) proxyfan alone has no significant effect on the food intake, suggesting in this behavioural system, proxyfan is having neither

an inhibitory or stimulatory effect at the H_3R. We have also demonstrated that when proxyfan was introduced to a brain slice via a perifusion bath, the neuronal firing rate of VMN neurones within the brain slice were unperturbed and remained at basal levels. Our data suggest that proxyfan is acting neither as an agonist or inverse agonist at the H_3R when administered alone in our extracellular electrophysiology system.

Gbahou *et al.* (2003) demonstrate that proxyfan can be a protean agonist at the H_3R, having a spectrum of activities ranging from potent agonist to potent inverse agonist. As a protean agonist it can produce different responses depending on the constitutive activity of the system being tested. Thus, it could be suggested that the lack of effect proxyfan had on food intake in both satiated and non-satiated animals is due to the equilibrium between the active and inactive conformations of the receptor.

As shown in this and in chapter 2 of this thesis, both H_3R drugs imetit and thioperamide have significant effects on food intake when given both at night or during the day and in satiated or fasting animals, with imetit causing a significant increase in food intake and thioperamide causing a significant decrease in food intake. Thus, it was interesting to find that proxyfan, which has no effects on food intake when administered alone, blocked any changes in food intake from basal levels when co-administered with either the H_3R agonist or inverse agonist. As proxyfan has no effect alone but can have effects on food intake when co-applied with imetit or thioperamide, proxyfan appears to be acting as a neutral antagonist in our feeding system. It also shows clearly that proxyfan can cross the blood-brain barrier to affect neuronal function, as has been shown in other studies. For example, proxyfan has been shown to affect memory (Cangioli *et al.,* 2002, Giovannini *et al.,* 2003), wakefulness (Gbahou *et al.,* 2003), or drinking (Fox *et al.,* 2002) in rats.

Our results from extracellular electrophysiology on histamine-responsive neurones within the rat VMN, suggests these neurones are unresponsive to proxyfan when it is administered alone, and is probably not acting as a H_3R agonist or inverse agonist in this system. We also have shown in previous chapters that thioperamide and imetit have an excitatory or inhibitory effect on neuronal firing within the rat VMN, respectively. Here we found that, when these drugs were co-applied with proxyfan, the excitatory effects of thioperamide or the inhibitory effects of imetit were blocked, suggesting proxyfan is antagonising the effects on histamine release mediated by presynaptic H_3R. Again, this suggests proxyfan is acting as a neutral antagonist.

By suppressing the constitutive activity at the H_3R, inverse agonists such as thioperamide enhance histamine release, whereas agonists such as imetit inhibit the release. Proxyfan alone has no effect on histamine release, but blocks the opposing effects of the H_3R drugs, thioperamide and imetit on both feeding and neuronal firing of histamine-responsive cells within the VMN, suggesting it is acting as a neutral antagonist in both systems. The lack of effect of proxyfan shows that antagonism of endogenous histamine does not contribute to the histamine-releasing effect of thioperamide and imetit.

The level of constitutive activity depends on both the number of spontaneously active conformations and the coupling efficiency of these conformations to G proteins (Arrang *et al.,* 2007). It therefore depends not only on the receptor, but also on the response. Inverse agonists impede constitutive activity by promoting inactive conformations. Their maximal effect is correlated to the level of constitutive activity. Thus, thioperamide is blocking the natural constitutive activity at the H_3R and promoting an inactive conformation which is in turn allowing the release of endogenous histamine. As Arrang *et al.* (2007) suggest proxyfan can act as an agonist when the agonist state promoted by proxyfan has an efficacy higher than the receptors constitutive state and it can act

as a inverse agonists when the state promoted by proxyfan has an efficacy lower than the receptor constitutive. But proxyfan alone will have no apparent activity when both the receptor constitutive state and the agonist state show the same efficacy, and thus here proxyfan is acting as a neutral antagonist (Arrang *et al.,* 2007). In our system, proxyfan is having no effect alone, but is able to block the effects of both imetit and thioperamide. This suggests both the agonist state promoted by proxyfan and the constitutively active state of the receptor are showing the same efficacy in our system and, thus, proxyfan is acting as a neutral antagonist.

Our results would agree with Morriset *et al.* (2000) who suggested the pharmacological profile of proxyfan depended on the system being analysed. Thus, the actions of proxyfan depend on the equilibrium between the active and inactive conformations of the receptor and the ratio of the receptor to the various G proteins.

As proxyfan is a protean agonist and we found it to act as a neutral antagonist in our system it suggest the H_3R agonist, imetit, and the H_3R inverse agonist, thioperamide act specifically at the H_3R. It important to note, that these results cannot be generalised to all neurons that are involved in appetite regulation. However, the consistency between my *in vivo* and *in vitro* data suggests that the effects myself and other research groups have noted in rodents are in fact real and the H_3R agonist, imetit and the H_3R inverse agonist, thioperamide, are truly specific for the H_3R. This adds further support to the vast majority of the literature (Jethwa *et al.,* 2009, Sindelar *et al.,* 2005, Lecklin *et al.,* 1998), and further brings into the question the validity of the Yoshimoto *et al.* (2006) study results described in Chapter 2 of this thesis, which found imetit and thioperamide to have the opposite effects on feeding to all other published research. Although it must be noted that it is difficult to disagree entirely with the findings of Yoshimoto and colleagues, as we did find imetit to have a similar effect

on feeding in mice as they illustrated. Also their experiments were carried out on H_3R knockout mice, which will have no endogenous H_3R tone and thus histamine may be unusually high in this system which could alter the data collected from adding H_3R agonists or H_3R inverse agonists.

Chapter 6:

Glucose tolerance and the histaminergic system

6.1 Introduction

As I have illustrated in this Thesis, a large population of VMN neurones are responsive to histamine, but these VMN neurones are presumably responsive to a number of other stimuli and, therefore, are able to integrate signaling related to energy homeostasis. Thus, these VMN neurones are possibly the same population that respond to leptin, ghrelin, and other metabolic signals. A major integratory role within the VMN is played by glucose-sensing neurones, but these only make up a relatively small proportion of the total number VMN neurones, and can respond differently to increases or decreases in glucose. Therefore, we wish to determine whether the same populations of VMN neurones responding to histamine are also glucose responsive.

6.1.1 Glucose sensing in the brain

Throughout this thesis I have presented evidence that the VMN is essential and plays a pivotal role in the regulation and maintenance of energy homeostasis. One important role is dependent on the high density of glucose-sensing cells within the VMN which will influence energy-regulating mechanisms (Gonzalez *et al.,* 2008). It is important to note that small changes in levels of glucose probably do not have a direct role in the timing or quantity of food intake, but the VMN is important in integrating both long- and short-term regulators of energy balance, and also in counter-regulatory mechanism to protect against hypoglycaemia. If the VMN detects hypoglycaemia, it initiates a number of immediate counter-regulatory mechanisms, the most important being the immediate release of glucose from the liver, which is mediated by the sympathetic nervous system. There is also a slower release of glucagon and inhibition of insulin secretion to counteract against hypoglycaemia. There is also an increase

in feeding in response to hypoglycaemia, but this is neither immediate nor important unless in extreme situations. Perhaps the more important function of the VMN in the acute regulation of food intake may be the ability of the VMN to reduce feeding in response to other stimuli.

Most brain glucose is used primarily as substrate for the energy needs of neurones and glia and does not alter the firing rate of the majority of neurones (Levin *et al.,* 1999). However, a small group of neurones within brain areas tied to the neuroendocrine and autonomic control systems appear to use glucose as a signal to alter their firing rate suggesting some neurones might be involved in energy homeostasis.

'Glucose-sensing' neurones are specialized cells that respond to small, physiological changes in extracellular glucose concentration by altering their firing rate (Burdakov *et al.,* 2005). Glucose-sensing neurones are present in a number of forebrain regions and the brainstem, but, like histamine responsive neurones, are most prevalent within the hypothalamus (Adachi *et al.,* 1995). Hypothalamic glucose-sensing neurones comprise groups of cells within the LH, ARC and VMN regions and exhibit both excitatory or inhibitory firing responses to changes in extracellular glucose concentration (Anand *et al.,* 1964; Oomura *et al.,* 1969; Routh VH., 2002; Wang *et al.,* 2004). The activity of these cells in response to the energy status of the body, can result in changes in hormone release, metabolic rate, food intake and locomotor activity to ensure the brain always has adequate glucose (Routh VH., 2002; Levin *et al.,* 2004; Routh *et al.*, 2004). This continual monitoring of brain glucose concentrations is critical as the brain becomes irreversibly damaged if deprived of glucose after only a few minutes.

Two populations of hypothalamic glucose-sensing neurones have been shown to exist, those excited and those inhibited by glucose (Burdakov *et al.,* 2005). Glucose-excited (GE) neurones and glucose-inhibited (GI) neurones are both located in the hypothalamic VMN, LH, ARC and PVN, and in the caudal

brainstem around the tract of the solitary nucleus (Song et al., 2001, Levin et al., 1999). The first evidence for the presence of hypothalamic GI neurones was provided by the *in vivo* experiments of Anand *et al.* who tried to correlate blood glucose levels with firing activity in the 'hunger' and 'satiety' regions of the hypothalamus (Anand *et al.* 1964). Using steel microelectrodes, they found that spike firing of LH neurones significantly decreased after intravenous infusion of glucose in anaesthetised dogs (Anand *et al.,* 1964). Oomura *et al.* also demonstrated that injection of glucose suppressed spike firing in 20% of LH neurones (Oomura *et al.,* 1969). Although GI and GE neurones both respond to glucose albeit in opposing manners, they are not similar in their physiology (Routh *et al.,* 2003).

Relatively little is known about glucosensing in GI neurons, but what is certain is that they respond to increasing glucose levels by decreasing their firing rate. Oomura *et al.* propose that glucose-inhibited neuronal activity is regulated by the Na^+-K^+-ATP pump (Oomura *et al.,* 1974). Whereas, Routh and colleagues obtained data indicating that they may use a Cl^- channel to sense glucose (Song *et al.,* 2001; Routh *et al.,* 2003). Because decreasing extracellular glucose levels should lower intraneuronal ATP levels, such a Cl^--channel should be responsive to changes in the ATP to ADP ratio. GE neurones show more similarity to pancreatic β-cells than GI neurons, and these similarities are proposed to account for how GE neurones sense glucose (Yang *et al.,* 1999). GE neurones increase their firing rate when ambient glucose levels rise and cease firing when glucose is removed (Oomura *et al.,* 1969). This response is thought to be modulated by a K^+ channel that is sensitive to the intracellular ratio of ATP to ADP. Thus, it is called the ATP-sensitive K^+ channel (K_{ATP}) (Trapp *et al.,* 1997). The K_{ATP} channel is inactivated by direct binding of ATP, whereas phosphorylation of the channel increases its activity (Routh *et al.,* 1997). Normal orexigenic responses to NPY and anorectic responses to leptin are maintained when K_{ATP} channel function is

interrupted, suggesting neuronal glucosensing is critical for glucoprivic, but not homeostatic ingestive behavior, and the ablation of K_{ATP} channel function attenuates the counter-regulatory response to hypoglycemia but does not alter basal plasma glucose levels (Miki *et al.,* 2001). It is suspected that glucose is only one of several metabolic signals sensed and integrated within energy-sensing neurones (Levin *et al.,* 2002).

6.1.2 *The VMN and glucose sensing*

Many glucose-sensing neurones are found in the classical 'satiety center' or VMN. Glucosensing neurones in the VMN are among the best characterized, with 14–19% of all VMN neurones being GE and 3–14% being GI in type (Levin *et al.,* 2004). Single-cell RT-PCR studies have suggested that some, if not all, VMN neurones including GE neurones, are GABAergic (Miki *et al.,* 2001; Kang *et al.,* 2004). Glucosensing neurones also express receptors for and respond to peripheral hormones that convey signals relating to fat stores such as leptin (Spanswick *et al.,* 2000) and insulin (Wang *et al.,* 2004). The sensitivity of the VMN GI neurones can be modulated by systemic hyperglycaemia (Canabal *et al.,* 2007) and hypoglycaemia (Song *et al.,* 2006), illustrating the importance of the VMN in relation to glucose sensing within the brain. The importance of glucose sensing within the VMN came about when data showed that mature rats with lesions in the VMN failed to display the preference reversal from concentrated to dilute glucose solutions that is seen in normal rats (Booth *et al.,* 1972; Jacobs *et al.,* 1958), indicating that the VMN could be involved in the sensing of metabolic signals arising from glucose. Panksepp and co-workers (1972) also established that rats with VMN lesions do not exhibit the prolonged depression of food intake after intraperitoneal glucose injections observed in control rats and in rats with lateral hypothalamic lesions (Panksepp *et al.,* 1972).

VMN GE neurones have been noted to be inhibited when extracellular glucose decreased to very low levels suggesting these neurones only respond to large decreases in extracellular glucose that accompany profound systemic hypoglycemia, rather than the relatively small changes in plasma glucose levels (Levin *et al.,* 1998). Thus, as with other GE neurones, VMN GE neurones are more likely to play a role in the counter-regulatory response to hypoglycemia (Yoshimatsu *et al.,* 1984) and glucoprivic feeding (Atef *et al.,* 1992), than in the control of meal initiation. A less common type of VMN neurone with inherent glucosensing properties are the GI neurones and which are found only in low abundance within the VMN. GI neurones are excited when extracellular glucose is decreased.

It is important to point out that, Song *et al.,* 2001 have shown that there are many neurones in the VMN that have no inherent glucosensing capacity of their own. Instead, their firing rate is regulated by presynaptic inputs from other glucosensing neurones either inside or outside of the VMN.

6.1.3 Histamine and glucose sensing

Histamine neurones in mammals project their efferent varicose fibres to almost all areas of the brain. The hypothalamus is one of the richest areas in density of histamine fibres, with histamine receptors being heavily localised in the hypothalamus (Sakata *et al.,* 1997). Although it must be pointed out that although histamine receptor expression is dense within the hypothalamus, it is far greater in other brain regions that are not related to energy homeostasis. Glucose-sensing neurones are found within the VMN and also tend to have receptors for other metabolic signals, such as leptin and insulin, demonstrating that glucose-sensing neurones are capable of integrating different types of signals. If these

same neurones are also responsive to histamine, then histamine may well be able to modulate glucose sensing, and therefore glucose handling.

Glucoprivation activates histamine neurones in the hypothalamus (Oohara *et al.,* 1994) and enhances glycogenolysis in the brain (Sakata *et al.,* 1994). Histamine neurones also accelerate lipolysis in adipose tissues to supply energy to the brain through activation of the sympathetic nervous system (Bugajski *et al.,* 1981). These findings regarding functional roles of histamine neurones suggest such systems are related to nutritional status and energy storage across a broad range, from starvation to hyperglycemia (Sakata *et al.,* 1997). Treatment of diet induced obese (DIO) and *db/db* obese mice with histamine lowers serum concentrations of glucose and insulin, improving glucose tolerance and insulin sensitivity (Masaki *et al.,* 2001).

6.2 Does thioperamide affect glucose metabolism?

Hyperglycemic responses to exogenous histamine have been shown (Nishibori *et al.,* 1987). Sakata *et al* measured the turnover of hypothalamic histamine after IP infusion of insulin and demonstrated that the turnover rate of hypothalamic histamine was accelerated (Sakata *et al.,* 1994). Sakata *et al.* suggest the accelerating effect of insulin on histamine release is mediated by hypoglycemia as ICV infusion of the glucose analogue 2-deoxy- glucose (2-DG) induces hyperglycemia and also increases the turnover rate of hypothalamic histamine (Sakata *et al.,* 1994, Sakata *et al.,* 1991). These results suggest a lack of neural glucose in the hypothalamus increases histamine turnover (Oohara *et al.,* 1994, Sakata *et al.,* 1994). Sakata and colleagues have shown hypothalamic histamine is activated and released in response to an energy deficit thus suggesting hypothalamic histamine may play a vital role in glucose utilization and

ensuring homeostatic maintenance of energy supplies in the brain (Sakata *et al.,* 1997). As shown in Chapter 2 of this thesis, thioperamide is a potent H_3R inverse agonist that causes significant reductions in food intake when given both centrally and peripherally. But thioperamide has also been shown to significantly increase plasma glucose in rats (Yoshimatsu *et al.,* 1993). Yoshimatsu *et al.* showed that thioperamide produced a hyperglycemic response through activation of endogenous histamine in rats (Yoshimatsu *et al.,* 1993). Thioperamide removes the normal feedback autoinhibitory control system of both histamine synthesis and release at the level of histamine nerve terminals thus increasing histamine neuronal activity and endogenous histamine release *in vivo* (Arrang *et al.,* 1987).

As it has been shown by a number of research groups that histamine turnover is accelerated and endogenous histamine release increases when glucose levels are low (Oohara *et al.,* 1994, Sakata *et al.,* 1994, Yoshimatsu *et al.,* 1993), we can assume that histamine plays a role in glucose utilization. Thioperamide, by blocking the autoinhibitory controls on histamine that the H_3R has it causes a increase in histamine release which could then go on to play a key role in glucose tolerance and consumption. We wanted to determine whether injecting the H_3R inverse agonist, thioperamide and, thus, increasing histamine release within the animal before injecting a bolus of glucose would affect the animals natural ability to partition glucose.

We carried out this experiment on 18 CD-1 male mice (7 weeks of age) and injected either 20 mg/kg of thioperamide of saline (100 µl injection volume) into the intraperitoneal cavity of the animal (n = 9 per group). Twenty minutes after the initial injection of either thioperamide or saline was given, we took the circulating blood glucose levels of the animal being tested by collecting blood from a small incision in the tail and using a One Touch Ultra 2 glucometer (LifeScan, Johnson-Johnson, UK). We then administered 2 g/kg of glucose into the intraperitoneal cavity. We took further circulating blood glucose readings, at

15, 30, 60 and 120 minutes post the injection of glucose. Blood glucose levels were presented as mmol/l and results were analysed and presented as mean ± SEM. Data from feeding groups were analysed using a two-way ANOVA with repeated measures and Bonferroni *post hoc* tests (see graph 7.1).

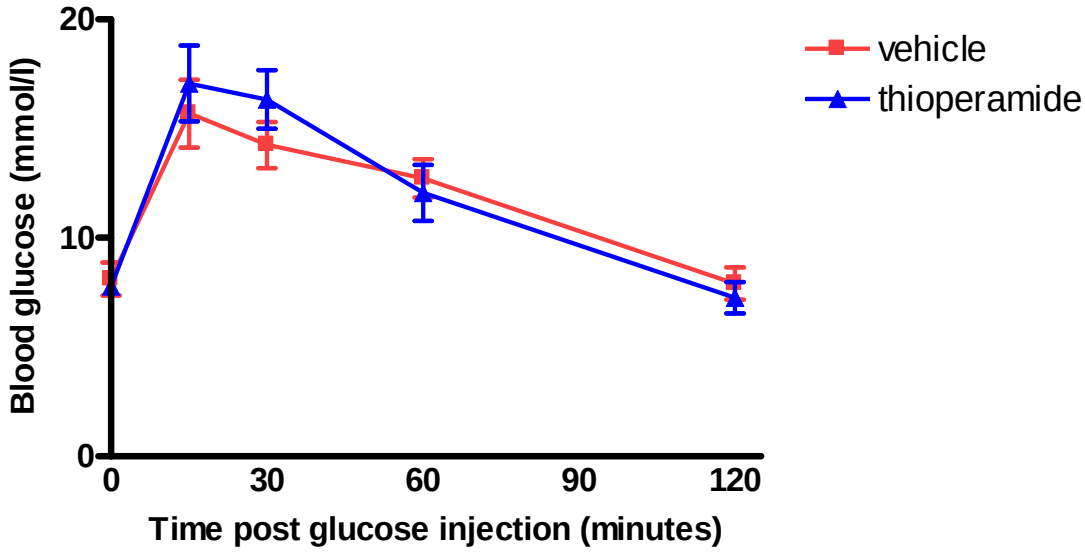


Figure 6.1.; Circulating blood glucose levels (mmol/l) measured at 15, 30, 60, 120 minutes after i.p. injection with glucose. (n = 9). Data are expressed as mean ± S.E.M. Two-way ANOVA with repeated measures and Bonferroni *post hoc* tests, no significance.

We found that thioperamide had no effect on glucose handling in the CD1 mouse (figure 6.1). Although thioperamide-treated animals did show some trend towards an increase in glucose, their blood levels returned back to basal levels faster than control group, and so there was no statistical significance. These results suggest that the increase in histamine resulting from the administration of thioperamide has little effect on the handling of glucose and is not associated with the anorexic effects of the H_3R inverse agonist. These preliminary results suggest

the actions of thioperamide are not directly affecting glucose sensing and metabolism, but further more in depth hormone studies are needed to determine the levels of compounds such as insulin and glycogen to determine whether histamine is affecting their levels when glucose levels vary. It also would be interesting to see what effect thioperamide has on glucose tolerance in obese models.

6.3 SF-1 neurones and possible role of PACAP

One of the major problems in studying the VMN is that is that we do not know the phenotype of the cells. Steroidogenic factor 1 (SF-1) is a member of the nuclear receptor family of intracellular transcription factors and is important in the development of the VMN. It regulates steroid hydroxylases and is a cell-specific nuclear regulator. Since SF-1 is only expressed in the VMN in the brain it can be used to target VMN neurones. Zhao and colleagues have demonstrated that targeted deletion of SF-1 results in the failed development of the VMN resulting in an obese animal (Zhao *et al.,* 2004). The SF-1 promoter has also been used to drive expression of Cre-recombinase, to selectively knock out “floxed” genes in the VMN. For example, SF-1-driven deletion of leptin receptors also results in an obese phenotype (Dhillon *et al.,* 2006). Many VMN neurones are glutamatergic, but less is understood about the neuropeptide populations in this area. Two possible neuropeptide candidates are pituitary adenylate cyclase-activating polypeptide (PACAP) and brain-derived neurotrophic factor (BDNF). These two neuropeptides appear to be in separate populations because most PACAP neurones in the adult mouse express SF-1, notably in the dorsomedial region of the VMN, but very few BDNF neurones in this region do (Hawke and Luckman, unpublished). Ideally, we would like to look at individual populations of cells within

the VMN, but that is not currently possible. Although SF-1 cells are probably a mixed population, Dhillon *et al.* (2006) suggest they mostly respond in the same direction and are excited by leptin, much as we would expect for histamine. Therefore, we wanted to determine whether SF-1 cells also contain a sub-population of glucose-sensing neurones. If they do, then our aim would be to see how these glucose-sensing neurones respond to histamine.

VMN cells are predominantly glutamatergic, like many hypothalamic neurones, and at least some of the effects of SF-1 cells in the VMN are mediated by glutamate (Tong *et al.,* 2007). One VMN-enriched gene is PACAP (Segal *et al.,* 2005; Kurrasch *et al.,* 2007), which plays a role in controlling both appetite and energy expenditure in mice (Morley *et al.,* 1992, Tachibana *et al.,* 2003, Matsuda *et al.,* 2005; Matsuda *et al.,* 2007). At least some of leptin's effects are mediated by this VMN PACAP population (Hawke *et al.,* 2009). Leptin signaling in the VMN plays an important role in regulation of energy homeostasis. Extracellular recordings have revealed that at least half of VMN neurones respond to the application of leptin (Irani *et al.,* 2008). Moreover, electrophysiological recordings from SF-1 neurones show the large majority are depolarized by leptin (Dhillon *et al.,* 2006). Electrophysiology and immunohistochemistry confirm that SF-1 cells in the VMN are a target for leptin, and that targeted genetic ablation of leptin receptors in the VMN results in obesity (Dhillon *et al.,* 2006). SF-1-Cre lepr flox/flox mice exhibit significantly less PACAP expression in the VMN than wildtype littermates (Hawke *et al.,* 2009).

As PACAP is clearly vital in VMN controlled feeding and the majority of leptin-sensitive neurones contain PACAP we hypothesis that the same population of cells could also be histamine-responsive and these cells could be mediating their affects on food intake via similar neuronal pathways.

6.4 How does glucose act SF1-positive neurones?

Extracellular electrophysiology determines the effects on a subset of neurones when a substrate is applied. The electrode will detect electrical activity generated by the neurones adjacent to the electrode tip, but the major downside to extracellular electrophysiology is that it is impossible to easily determine the identity of the neurones being recorded. Also, extracellular recordings may be generated by the collective activity of many cells and are affected by the simultaneous activation of many neurones by synaptic transmission. With single-cell, patch-clamp electrophysiology, you can either fill the recorded cell with a marker and identify it post-recording using immunocytochemistry or, as done here, record from pre-identified neurones. This is possible because the SF1-Cre mouse we have used was originally crossed with a green-florescent protein (GFP) reporter mouse, so that GFP in the offspring is now expressed only in SF-1 neurones (Dhillon *et al.,* 2006). Thus, with help from Dr Mino Belle, we have preliminary data to begin the task of phenotyping the VMN neurones that are sensitive to glucose and histamine.

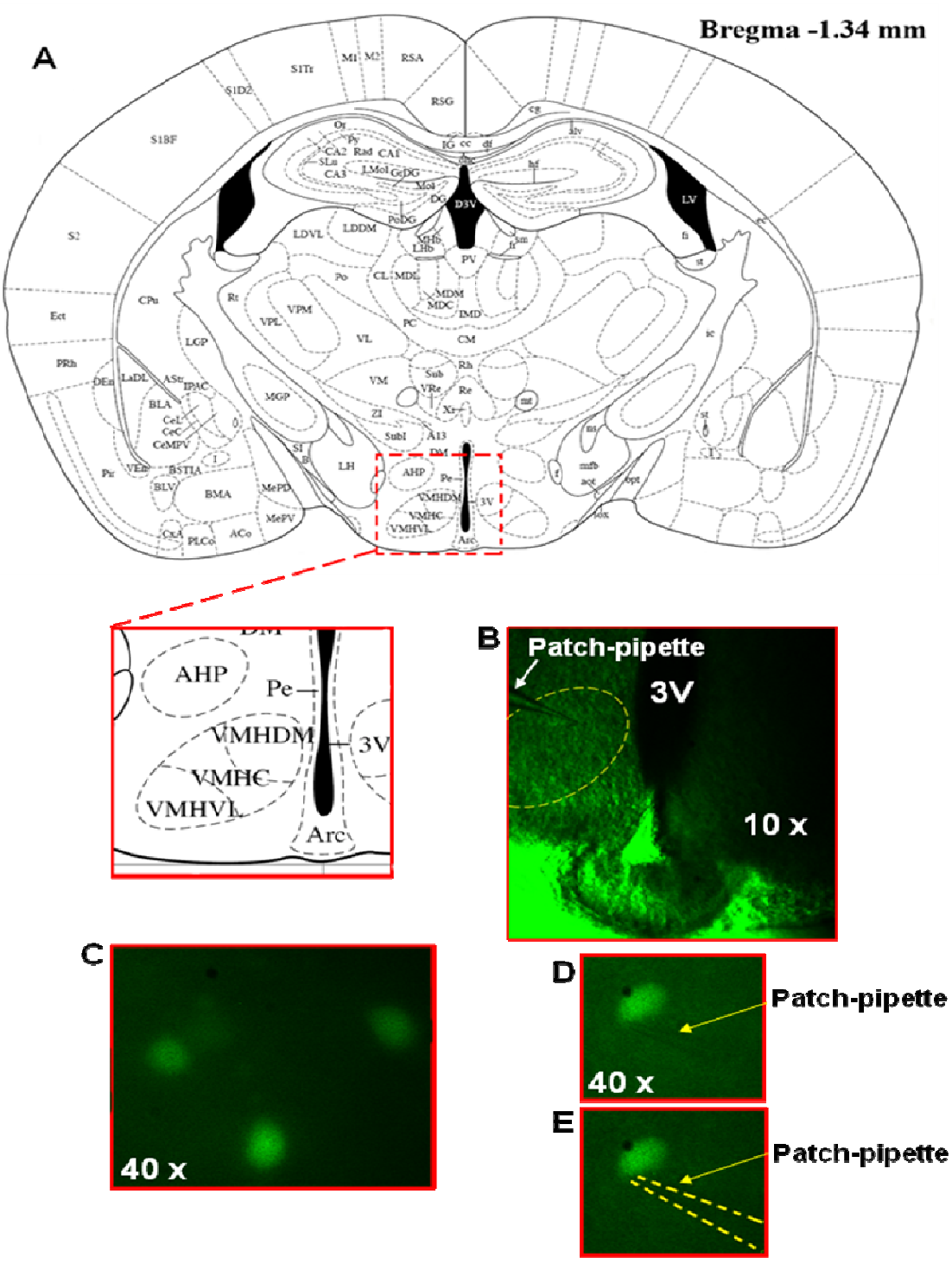
To determine how glucose affects neuronal firing within the VMN we measured changes in the excitability of VMN neurones *in vitro* using whole-cell patch recordings from SF-1 VMN neurones pre-identified by their expression of GFP. Mice (7-9 months old) expressing GFP driven by the SF-1 gene in the VMN were bred and group housed on a 12:12 hour light:dark cycle with lights on at 8:00 h. Animals were fed *ad libitum*. Animals were terminally anaesthetized with isofluorane and killed by decapitation. The brain was immediately removed and coronal brain slices containing the VMN were collected using a Vibroslicer (Campden Instrument, Loughborough, Leicestershire, UK) into an ice-cold low Na^{+}/Ca^{2+}, high Mg^{2+} sucrose-based incubation artificial cerebro-spinal fluid (aCSF) (in mM: NaCl 95; KCl 1.8; KH2PO4 1.2; CaCl2 0.5; MgSO4 7; NaHCO3

26; glucose 15; sucrose 50; Phenol Red 0.5mg/l; oxygenated with 95% O_2; 5% CO_2; pH 7.4, measured osmolality 300-310 mosmol kg^{-1}). For patch-clamp recordings, slices were transferred to a recording bath and were continuously perifused (1.5-2.5 ml min^{-1}) with oxygenated recording aCSF at room temperature. The recording aCSF was identical to the incubation solution except for the following (mM): NaCl 127; CaCl2 2.4; MgSO4 1.3; sucrose 0. Slices were allowed to recover for at least 1 hour prior to whole-cell recording. Cell membrane was ruptured with or without minimum holding currents using a patch pipette electrode (7-10 MΩ), made from thick-walled borosilicate glass capillaries (Harvard Apparatus Ltd, Kent, UK) using a two-stage vertical micropipette puller (Narashige, Tokyo, Japan). Signals were sampled at 30 kHz, stored and analyzed using Spike2 software (Cambridge Electronic Design, Cambridge, UK). All data acquisition and step protocols were generated through a micro 1401 mkII interface (CED). GFP neurones were visually identified using a Leica epifluorescent microscope (DMLFS; Leica Microsystems Ltd, Milton-Keynes, UK) equipped with filters optimized for visualizing GFP through a 40x water-immersion lens (HCX APO). Neurones were allowed to recover for 10-20 seconds, and all data were collected within 4 minutes of cell membrane rupture to minimize any potential cell dialysis effects (see Belle *et al.,* 2009 for more details on the setting up and recording of whole-cell patch-clamp electrophysiology).

To determine the cell's response to increased glucose we firstly bath applied 1 mM glucose solution mixed with fresh aCSF for 1 minute to determine the cells response to low glucose and then bath applied 5 mM of glucose solution mixed with fresh aCSF for 5 minutes and recorded the changes in membrane potential and neuronal firing.

Unfortunately due to the lack of SF-1-Cre lepr wt/wt mice, genotyping drawbacks and time restrictions we were only able to collect recording from a few cells, only one of which was intrinsically glucose sensitive.

Figure 6.2.; This diagram (see next page) illustrates the area within the mouse hypothalamus we recorded from and allows us to demonstrate the GFP identified SF-1 cells within this area. A. illustrates the dorsomedial ventromedial hypothalamus (VMHDM) where our electrode was placed, B. illustrates a low magnification of the VMHDM area and demonstrates the GFP within the SF-1 cells in this area, C. demonstrates the SF-1 cells expressing GFP under a high magnification which allowed us to specifically record from this subset of cells using whole cell-patch clamp electrophysiology, D. illustrates our glass pipette electrode recording from a SF-1 GFP expressing cell, E. illustrates more clearly the glass pipette electrode recording specifically from a SF-1 cell. The yellow dotted lines indicate the edges of the glass electrode.


A
Bregma -1.34 mm
AHP
Pe
VMHDM
3V
VMHC
VMHVL
Arc
B
Patch-pipette
3V
10 x
C
40 x
D
Patch-pipette
40 x
E
Patch-pipette

As discussed in the introduction of this chapter, both GE and GI neuronal populations are found within the VMN, with a slightly higher population being glucose excited. We hypothesized that as more VMN glucose responsive cells are found to be GE than GI we would expect to find cells that increased their neuronal firing when glucose was added. The one recording we obtained, was found to be a glucose-inhibited neurone. As can be seen in figure 6.3 when the higher concentration of glucose was added (5 mM) we recorded a gradual hyperpolarisation of 10 mV in the membrane potential, which eventually resulted in an inhibition of cell firing. The cell did begin to return to basal membrane potential levels around 30 minutes after the high-glucose medium was thoroughly washed off, showing this was a reversible decrease in firing. The membrane properties of this neurone (results not shown), suggested that it had similar properties to glucose-inhibited neurones recorded in the lateral hypothalamus (Burdakov *et al.,* 2005).

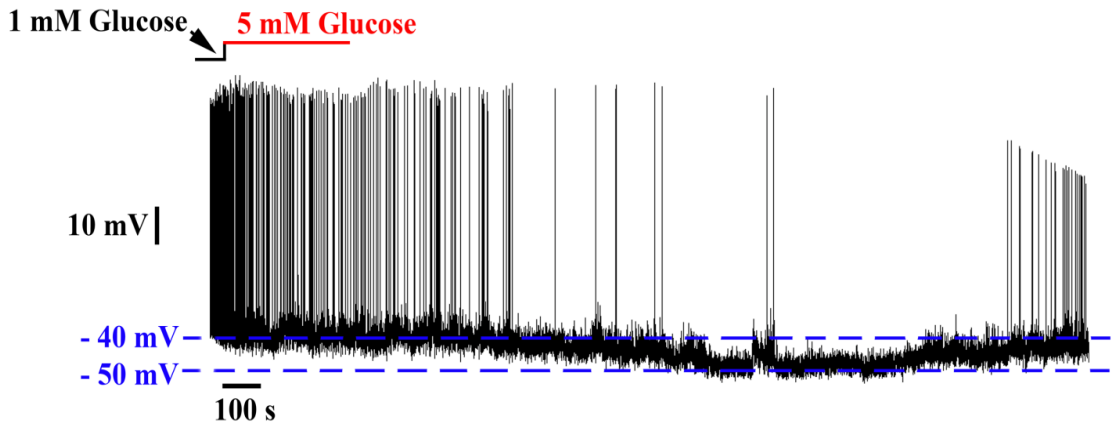


Figure 6.3.; This patch clamp electrophysiology trace shows the change in membrane potential the SF-1 GFP VMN cell we recorded from demonstrated when the glucose concentration within the aCSF solution was increased from 1 mM to 5 mM. There is a gradual lowing of the membrane potential seen after the higher concentration of glucose is added suggesting this particular VMN cell is glucose-inhibited. This recording was carried out by Dr Mino Belle.

6.5 Future work

The regulation of feeding by histamine and glucose sensing both involve the VMN. We began to investigate whether glucose sensing interacted with histaminergic signaling within the VMN. Unfortunately due to a number of setbacks and a lack of time, we were only able to carry out very preliminary studies.

With the help and expertise of Dr Mino Belle we demonstrated glucose sensing by at least some SF-1 cells within the dorsomedial VMN. Obviously as only one recording was obtained, we would plan to carry out more recordings on this population of SF1-positvie cells and determine whether the majority are glucose inhibited or glucose excited.

Once the population of glucose responsive cells has been characterized, we would then aim to determine whether these cells also are influenced by the histaminergic system. To determine this we would apply the H_3R inverse agonist thioperamide which would increase histamine release within the slice and see if thioperamdie modified the firing patterns of glucose-sensing neurones.

We found thioperamide had no significant effect on the glucose tolerance of CD1 mice when given before a bolus injection of glucose, though there may have been a slight increase in circulating glucose as suggested in other reports. It is too early to determine if histamine is interacting directly in the VMN to modify glucose handling. Thus, one further study would be to determine the effects of thioperamide on glucose tolerance in obese mice. We could also measure insulin and glucagon responses in the blood of mice that received thioperamide.

Chapter 7:

General Discussion

General Discussion

The key aims of this PhD thesis were to determine the involvement of the histaminergic system in relation to food intake and appetite control and whether the H_3R could, like the H_1R, be a possible target for the development of pharmacological products to advance the fight against obesity. We also wanted to determine where in the rat brain H_3R ligands were acting and if they were acting directly on known 'feeding' and 'satiety' centres within the hypothalamus. We aimed to establish what effects histamine has on neuronal firing within the rat VMN, including whether H_1 or H_3 receptors are present within the rat VMN and whether the drugs we tested in *in vivo* feeding studies also altered neuronal firing in this area, which is known to be pivotal in the regulation of feeding. Our final aim was to ensure our H_3R drugs, imetit and thioperamide, were acting specifically at the H_3R.

7.1 The histaminergic system can be pharmacologically manipulated to affect food intake

Histamine is an important central neurotransmitter and is involved in a large number of functions including arousal, cognition, locomotor activity, autonomic and vestibular functions, feeding and drinking, sexual behaviour and analgesia (Hough LB., 1988; Schwartz *et al.,* 1991; Wada *et al.,* 1991, Chotard *et al.,* 2002, Gomez-Ramirez *et al.,* 2002, Hancock *et al.,* 2004). A number of research groups have demonstrated the importance of histamine in controlling food intake and all have suggested it is a potent anorexigenic agent (Yoshimatsu *et al.,* 1993, Yoshimatsu *et al.,* 2002, Yoshimatsu H., 2006, Yoshimoto *et al.,* 2006, Gotoh *et al.,* 2007). For example, behavioural studies have illustrated that histamine suppresses food intake when administered both centrally or systemically (Doi *et*

al., 1994, Lecklin *et al.,* 1998, Lecklin *et al.,* 1995, Endou *et al.,* 2001). We have demonstrated that when histamine is administered centrally into the rat it results in a rapid, yet short-lasting, highly significant reduction in food intake. These results concur with other reports suggesting histamine is a potent hypophagic agent.

Of the four documented histamine receptors only the H_1R and H_3R have been found to be involved in the regulation of feeding and obesity in rodents (Masaki *et al.,* 2006). H_1R activation causes a reduction in food intake and, thus, pharmaceutical agencies first looked to establish drug therapies that targeted this H_1R as a treatment for weight gain. The main issue with targeting the H_1R is that it is involved in a large number of bodily functions and it is distributed widely throughout peripheral tissues, as well as the CNS (Kinnunen *et al.* 1998). Thus, altering the activity of this receptor will not specifically target just one of its many functions, but will result in a number of adverse effects, including drowsiness. Therefore, we focused our attention on the H_3R which has been illustrated by a number of reports to have potential anti-obesity effects, though the exact therapeutic benefits remain controversial. For example, thioperamide, a potent H_3R inverse agonist should theoretically reduce food intake by increasing endogenous histamine levels which then activate the H_1R resulting in a reduction in food intake. This has been found by some research groups (Lecklin *et al.,* 1998, Sindelar *et al*., 2004), but others found it to have no effect (Itoh *et al.,* 1998), and some even found giving thioperamide increased food intake and weight gain in mice (Yoshimoto *et al.,* 2006). Therefore, we wanted to determine what effect on food intake a H_3R agonist and two H_3R inverse agonists would have on food intake in our chosen rat model. We found that the H_3R agonist imetit, caused a significant increase in food intake, whereas the H_3R inverse agonists thioperamide and NNC1202, caused dramatic hypophagia in the rat. Using analysis of the behavioural satiety sequence, we found that imetit and

thioperamide appeared to change feeding without causing any unexpected, adverse effects. By contrast, NNC1202 caused the animals to withdraw and become inactive, displaying “sickness-like” behaviour, which explains the dramatic and prolonged reduction in food intake caused by non-selective actions.

We also wanted to determine whether feeding in mice and rats were equally affected by imetit and thioperamide, or whether these drugs, as suggested by some research groups (Tokita *et al.,* 2006, Yoshimoto *et al.,* 2006), exerted species-related differences in their actions and abilities to alter feeding. We found that both imetit and thioperamide caused a significant reduction in food intake in mice. This suggests that thioperamide can cause hypophagia in both rats and mice and, thus, inverse agonists at the H_3R remain a potential target for anti-obesity therapies. The effects of imetit on the other hand appear to alter depending on the species of animal being investigated. The reasons for these differences are currently unknown, but may reflect differences in H_3R splice variants expressed in different areas of the CNS or in different species. Splice variant may interact differently with pharmacological reagents such as imetit and thus result in distinct outcomes in different species.

7.2 The hypothalamus is a key target for the histaminergic system

The hypothalamus is a key brain area identified as having an important role in controlling food intake and energy expenditure (King BM., 2006). From brain lesion studies, the VMN and PVN are regarded predominantly as satiety centres, and the LH as a feeding centre. But the ARC, DMN and SCN all play important roles in controlling appetite as well (Beck *et al.,* 2000). Histamine is known to reduce food intake, but where and on what neuronal types it is acting has not been fully elucidated. Histaminergic nerve fibres are found in virtually all parts of

the brain but with especially high densities being apparent within the posterior hypothalamus, including in particular the VMN, SCN and PVN, although all hypothalamic areas receive a fairly strong histaminergic innervation with histaminergic fibres (Gomez-Ramirez *et al.,* 2002). The H_1R is found throughout the mammalian PNS and CNS but has specifically been shown to be present within the VMN and PVN (Masaki *et al.,* 2003). It is widely accepted that histamine acts via the H_1R to reduce food intake. We found using c-Fos, the neuronal marker for activation, that cells were activated within the hypothalamus of the rat brain. In particular, there was increase neuronal activation within the VMN, PVN, ARC, LH and DMN, all known to play a part in the regulation of appetite. As H_1R are present within the rat hypothalamus, histamine acts through the H_1R to reduce food intake there is increased neuronal activation within this area, we could assume that histamine is acting either directly or indirectly through the VMN, PVN, ARC, LH and DMN to have its hypophagic effects.

There is a high density of H_3R within the mammalian hypothalamus, striatum and nucleus accumbens **(**Hussain *et al*., 2002). In particular, H_3R mRNA have been detected in a number of areas within the hypothalamus including the VMN and the ARC (Lovenberg *et al.,* 1999). Although the highest densities of H_3R can be found in the CNS, they have also been found to be present, albeit in much lower levels, within the PNS, for example, in the GI tract, the airways and the cardiovascular system (Celanire *et al.,* 2005). In much the same manner as histamine, the application of the H_3R agonist, imetit, and the H_3R inverse agonists, thioperamide and NNC1202, caused increased neuronal activation within the same key feeding centres of the rat brain. H_3R have been shown to be present within the hypothalamus of the rat brain, in particular the VMN and ARC (Lovenberg *et al.,* 1999), It is widely assumed that H_3R inverse agonists and antagonists should affect food intake by blocking the negative feedback mechanisms of the H_3R that control the synthesis and release of histamine and

by doing so a increase in histamine is seen which then could go on to activate hypothalamic H_1R resulting in anorexia. It is important to point out here that due to the high level of constitutive activity at the H_3 receptor, the receptor has intrinsic activity even without the action of a ligand, and hence why the H_3R inverse agonist thioperamide is effective here. H_3R agonists on the other hand could be causing a reduction in endogenous histamine resulting in hyperpagia. It is important to point out here that as H_3R can reside on the terminals of neurones containing other neurotransmitters that themselves affect feeding behaviours, this is another possible mechanism which may result in the changes observed on food intake.

7.3 H_1 and H_3 receptors are present within the rat VMN

The VMN plays a crucial role in regulating food intake. It has been demonstrated in lesion studies and clinical observations that damage to the VMN is associated with increased food intake and morbid obesity (Anand *et al.,* 1951). The VMN is referred to as the "satiety centre" of the brain and therefore, if damaged, it can no longer enforce feeding restraints resulting in a overeating and weight gain (Sclafani, 1971, Bray *et al.,* 1981). The VMN has direct links with the PVN and DMN and via these it connects indirectly with the LH (Harrold *et al.,* 2004), illustrating the complex circuitry surrounding the VMN and the importance it carries in regulating food intake within the hypothalamus. VMN lesion-induced obesity has been found in both rodents (Mayer *et al.,* 1955) and humans (Bray *et al.,* 1975) demonstrating the importance of researching this area when looking for pharmacological targets against obesity. The VMN is reported to be the preferential site of histamine-mediated suppression of food intake in the mammalian brain (Haas *et al.,* 2008) and, thus, we aimed our electrophysiology

research the rat VMN. We found that approximately two-thirds of VMN cells responded to histamine with an increase in firing.

Histamine causes a reduction in food intake via the H_1R most probably within the VMN thus we could assume this increase in neuronal firing is due to the H_1R. Therefore, we co-administered a H_1R antagonist, pyrilamine and blocked histamine's excitatory affects on neuronal firing. This provides further evidence to support the presence of the H_1R within the rat VMN. Ookuma *et al.* (1993) had suggested H_1R have a suppressive role on feeding because these receptors are distributed more densely in the nuclei involved in the suppressive function of food intake, such as the VMN.

There is much less research identifying the distribution of the H_3R within the rat brain compared to the H_1R, but it is suggested that the H_3R can regulate histamine release within the VMN, potentially through its autoinhibitory pathways. Using the H_3R inverse agonist thioperamide, we were able to demonstrate the presence of H_3R in or near the VMN. By co-applying the H_1R antagonist, pyrilamine with thioperamide, we were also proved that the H_3R population in the VMN are autoreceptors as opposed to heteroreceptors. Our results collective results strongly suggest that H_1R andH_3R present in the VMN have a key role in the regulation of appetite.

7.4 Thioperamide and imetit are acting specifically at the H_3R

The H_3R is a 7TM GPCR with a high level of constitutive activity (Gbahou *et al.,* 2003). Using the fact H_3R can act constitutively to our advantage, we aimed to determine whether the H_3R agonist, imetit, and the H_3R inverse agonist, thioperamide, were acting specifically at the H_3R to have their effects on feeding and neuronal firing. GPCRs are allosteric proteins that adopt inactive and active

conformations in equilibrium. The active conformation is promoted by agonists, such as imetit, or can occur spontaneously, leading to constitutive activity of the receptor. Alternatively, inverse agonists, such as thioperamide, promote an inactive conformation and decrease constitutive activity (Gbahou *et al.,* 2003). Constitutive activity refers to the synthesis of a protein or an enzyme at a constant rate regardless of physiological demand or the concentration of a substrate. Compounds that bind to GPCRs without altering the equilibrium between active and inactive states of the receptor are referred to as neutral antagonists (Milligan *et al.,* 2003). Some ligand can act as protean agonists. One such compound is proxyfan, which depending on the system it is introduced to can act as an agonist, antagonist or neutral antagonist depending on the level of constitutive activity. We have shown that in both of *in vivo* studies looking at proxyfan's effects on feeding and in our *in vitro* system investigating proxyfan's effect on VMN neuronal firing, proxyfan alone has no effect. But we demonstrated that proxyfan can block the actions of both the H_3R agonist imetit and the H_3R inverse agonist thioperamide both *in vivo* and *in vitro*. Therefore, in the feeding circuitry it appears that proxyfan is acting as a neutral antagonist. As proxyfan is a protean agonist, we could use it as a tool to determine the ligand-specific nature of our imetit andthioperamide. We found that imetit and thioperamide were acting specifically at the H_3R as proxyfan, when co-administered with the H_3R agonist and H_3R inverse agonist, blocked the actions of both these compounds.

7.5 Conclusion

This PhD thesis has investigated the histaminergic system and if such a complex signaling system could be manipulated pharmacologically to have a beneficial effect on feeding behaviours. We have shown that histamine itself is a

potent anorexigenic agent and by focusing our initial investigations on H_3R compounds, we have proven that the H_3R inverse agonist thioperamide causes significant hypophagia in both rats and mice. However, imetit, an H_3R agonist, produces significant hyperphagia in rats whilst it reduced feeding in mice. Others have postulated that certain H_3R drugs can have opposing effects on food intake depending on the animal model being tested, suggesting species-related differences could pose a problem for anti-obesity drug development.

We have also illustrated the key areas activated by histamine and the H_3R-selective compounds tested, highlighting the feeding and satiety centres known to play a vital role in controlling food intake. This suggests possible areas or action that histamine, imetit, thioperamide and NNC1202 include the VMN, DMN, ARC, PVN and LH all integral in the control of appetite. Unfortunately, the coded compound, NNC1202, also appeared to have very strong adverse effects, which precludes it from future development.

We have shown that both H_1R and H_3R are present within the VMN of the rat brain and that histamine produces an excitatory neuronal firing response via both these receptors. We have also concluded that H_3R are autoreceptors within the rat VMN. As the VMN plays one of the most fundamental roles in the regulation of feeding and we have shown a population of both H_1 and H_3 receptors within this area, this is an important site for the histaminergic regulation of feeding.

Finally we have proven, using a neutral antagonist that the main H_3R compounds tested during this PhD were acting specifically via the H_3R in rats and, thus, allows us to conclude confidently that the effects of both imetit and thioperamide on feeding and neuronal VMN firing were a direct result of their efficacy at H_3R.

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