

RESEARCH PAPER

Proxyfan acts as a neutral antagonist of histamine H₃ receptors in the feeding-related hypothalamic ventromedial nucleus

RH Clapp and SM Luckman

Faculty of Life Sciences, University of Manchester, Manchester, UK

Correspondence

Professor Simon Luckman,
Faculty of Life Sciences,
University of Manchester, A.V.
Hill Building, Oxford Road,
Manchester M13 9PT, UK. E-mail:
simon.luckman@manchester.ac.uk

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BACKGROUND AND PURPOSE

Centrally acting histamine H₃ receptor ligands are proposed as potential treatments for obesity, although the value of inverse agonists at these receptors is still debated. Functional inhibition of H₃ autoreceptors activates neurones in a hypothalamic 'satiety' centre. The H₃ receptor antagonist, proxyfan was used as a tool to assess the action of histaminergic compounds in this model.

EXPERIMENTAL APPROACH

We compared the actions of histamine on feeding with those of an H₃ receptor agonist (imetit) and inverse agonist (thioperamide) in rats and mice. Sites of action were identified by immunohistochemistry and the hypothalamic ventromedial nucleus (VMN) was investigated using electrophysiological techniques.

KEY RESULTS

Central histamine or thioperamide decreased fast-induced feeding, whereas imetit increased feeding. Systemic thioperamide entered the brain to activate hypothalamic feeding centres and to reduce feeding without causing any adverse behaviours. Thioperamide activated neurones in the VMN through an action on histamine autoreceptors, whilst imetit had the opposite effect. Proxyfan administered alone did not affect either feeding or electrical activity. However, it blocked the actions of both thioperamide and imetit, acting as a neutral antagonist in this system.

CONCLUSIONS AND IMPLICATIONS

The H₃ receptor inverse agonist, thioperamide, potently reduced appetite without adverse behavioural effects. This action was blocked by proxyfan, acting as a neutral antagonist in this model and, therefore, this compound is useful in determining the selectivity of H₃ receptor-directed drugs. A major action of thioperamide is through presynaptic autoreceptors, inducing stimulation by endogenous histamine of postsynaptic H₁ receptors on anorectic hypothalamic neurones.

Abbreviations

aCSF, artificial cerebrospinal fluid; ARC, arcuate nucleus; DMN, dorsomedial nucleus; LHA, lateral hypothalamic area; NGS, normal goat serum; PVN, paraventricular nucleus; VMN, ventromedial nucleus of the hypothalamus

Introduction

Histamine is a multifunctional amine messenger, implicated to function in many body systems. Within the brain it is important in arousal, cognition, the autonomic and neuroen-

docrine systems, and has a strong influence on ingestive behaviour (Haas *et al.*, 2008). Inhibitors of the synthetic enzyme, histidine decarboxylase, or histamine H₁ receptor antagonists increase food consumption (Fukagawa *et al.*, 1989; Orthen-Gambill and Salomon, 1992; Ookuma *et al.*,

1993; Lecklin *et al.*, 1998; receptor nomenclature follows Alexander *et al.*, 2011). By contrast, increasing the availability of endogenous histamine suppresses appetite (Sheiner *et al.*, 1985; Ookuma *et al.*, 1993; Malmlof *et al.*, 2007; Ishizuka *et al.*, 2008). The level of histamine in the hypothalamus increases with appetitive behaviour (Itoh *et al.*, 1991), and the mediobasal region of the hypothalamus has been proposed as a site of action following region-specific injections of histaminergic drugs (Sakata *et al.*, 1988; 1990; Fukagawa *et al.*, 1989). Specifically, the ventromedial nucleus (VMN) of the hypothalamus has long been known as an important 'satiety' centre from classic brain-lesioning studies, and to be the region most densely populated with glucose-sensing neurones (King, 2006). Thus, the VMN has important roles in monitoring and responding to both acute and longer-term changes in energy status. It is a major site for the action of the adipose cell-derived hormone, leptin (Dhillon *et al.*, 2006), as well as a key region for countering hypoglycaemia (Borg *et al.*, 1997). Despite its importance in such key homeostatic mechanisms, still relatively little is known about the phenotypes of the neurones involved, although pituitary adenylate cyclase-activating polypeptide (Hawke *et al.*, 2009), brain-derived neurotrophic factor (Unger *et al.*, 2007) and glutamate-containing (Tong *et al.*, 2007) neurones are clearly involved.

Because of the marked effects of histamine and H_1 receptors on peripheral targets, including the immune system (Haas *et al.*, 2008), it would be impossible to contemplate compounds acting on H_1 receptors that could act selectively only on central circuits involved in body weight or glucose homeostasis. However, because the H_3 receptor is expressed predominantly within the CNS, compounds acting on these receptors have been developed with the view to targeting feeding behaviour (Hancock and Brune, 2005; Leurs *et al.*, 2005). The H_3 receptor is mainly a presynaptic receptor, expressed both on histaminergic neuronal cell bodies and terminals in many areas of the brain (Lovenberg *et al.*, 1999; Pillot *et al.*, 2002) and also on heterologous synapses (including those containing other amine transmitters (Arrang *et al.*, 1983; Leurs *et al.*, 2005). Importantly, the H_3 receptor also shows a high level of constitutive activity, showing that there is a strong endogenous 'brake' on the central release of histamine in regions where the receptors are located (Morisset *et al.*, 2000; Arrang *et al.*, 2007). With this discovery, many H_3 receptor antagonists were reclassified as inverse agonists capable of negatively regulating adenylate cyclase through binding $G_{i/o}$ proteins (Arrang *et al.*, 1983; Lovenberg *et al.*, 1999; Wulff *et al.*, 2002). The vast majority of published data indicate that H_3 receptor inverse agonists decrease food intake in rats (Itoh *et al.*, 1998; Lecklin and Tuomisto, 1998; Lecklin *et al.*, 1998; Hancock *et al.*, 2005; Malmlof *et al.*, 2005; 2006), mice (Hancock *et al.*, 2004; 2005), hamsters (Jethwa *et al.*, 2009), pigs and primates (Malmlof *et al.*, 2007), whilst agonists increase feeding (Kent *et al.*, 1997; Chiba *et al.*, 2009; Jethwa *et al.*, 2009). However, there are other papers that have found varying efficacy (Merali and Banks, 1994; Itoh *et al.*, 1998; Sindelar *et al.*, 2004), and one paper that found opposing effects with compounds acting on H_3 receptors (Yoshimoto *et al.*, 2006).

An exact understanding of how histaminergic drugs act in hypothalamic feeding circuits has been hampered by the

lack of good neutral antagonists. Proxyfan is classified as an antagonist with a K_i of 1–5 nM at H_3 receptors which is more than 1000-fold lower than those for other histamine receptors (Ligneau *et al.*, 1994; Morisset *et al.*, 2000; Gbahou *et al.*, 2003). However, as with a number of G protein-coupled receptors, H_3 receptors can exhibit different levels of constitutive activity *in vivo* and proxyfan has the potential to act pharmacologically through a spectrum of potencies (Arrang *et al.*, 2007). As constitutive activity does vary, it is important to determine the specific actions of H_3 receptor ligands in the system that is being manipulated. Proxyfan has been used relatively infrequently in whole animal studies. It was a H_3 receptor agonist in contextual fear memory formation (Baldi *et al.*, 2005), a potential inverse agonist in arousal and glucose handling (Gbahou *et al.*, 2003; Henry *et al.*, 2011) or a neutral antagonist in drinking behaviour (Fox *et al.*, 2002). Here, using the rat as our model, we showed, *in vivo*, that the VMN is a major target for compounds acting on histamine receptors that affect feeding behaviour. *In vitro* electrophysiology demonstrates the sensitivity of the majority of neurones in the dorsomedial region of the VMN to an excitatory action of histamine through H_1 receptors and that this pathway can be modulated locally by presynaptic H_3 autoreceptors. We show that proxyfan acts as a neutral antagonist in this system, blocking the actions of both H_3 receptor agonists and inverse agonists. Furthermore, proxyfan has the same effects *in vivo* to block the actions of other compounds acting on H_3 receptors, without affecting feeding behaviour itself.

Methods

Animals and feeding experiments

All animal care and experimental procedures complied with the Home Office (Animals) Procedures Act (1986) and were approved after local ethical review. The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010).

Outbred, male Sprague-Dawley rats (225–275 g) or CD1 mice (25–30 g; both from Charles River, Margate, UK) were used (total = 290). Animals were kept in a 12 h light/12 h dark cycle (lights on 0800–2000) within the University of Manchester animal facility for at least 1 week prior to the start of each experiment. The animals were housed in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) with a relative air humidity of 40–60%, and with free access to food (Beekay, Hull, UK) and water. Animals were housed singly to allow measurement of normal, night-time food intake, or overnight fast-induced re-feeding. Drugs were administered either *i.p.* (volumes of $1 \text{ mL}\cdot\text{kg}^{-1}$ body weight in isotonic saline) or *i.c.v.* ($2 \mu\text{L}$ per animal). Immediately following drug administration, weighed food was presented and intake measured at 1, 2, 4, 12 and 24 h later.

For the placement of in-dwelling *i.c.v.* cannulae, rats were anaesthetized with 2% isoflurane (Concord Pharmaceuticals Ltd, Reading, UK) in O_2 at $1 \text{ L}\cdot\text{min}^{-1}$. A 21-gauge guide cannula was implanted 0.8 mm posterior and 1.5 mm lateral to bregma, and 3 mm into the brain to access the lateral

ventricle (Paxinos and Watson, 1986). This was fixed to the skull with acrylic dental cement (Simplex Rapide, Austenal Dental, Hoorn, the Netherlands) adhered to two jeweller's screws. For post-operative analgesia, rats were injected with $10 \mu\text{L}\cdot\text{kg}^{-1}$ buprenorphine (Vetergesic, Reckitt Benckiser Healthcare, Hull, UK) and a bolus of saline to aid recovery, and left for 1 week before experimentation.

To measure the behavioural satiety sequence, rats ($n = 8$) were housed singly, 2 days before and then throughout the experiment, in transparent observation cages. Treatments were randomized. Food was removed 2 h before the experiment began to ensure no pre-feeding occurred. Each animal received one injection of each treatment with a minimum of 3 days between each experiment. After the drug was administered, rats were given a pre-weighed amount of food and behavioural observations began 90 s later. The animals were scored every 30 s over a 90-min period, with the behaviour recorded as either feeding, drinking, grooming, resting, inactive or active (Dodd *et al.*, 2010). The data were collated into 5-min period bins and expressed as the mean percentage of total behaviour for each animal and then for the treatment. In addition, the amount of food eaten by each animal over the 90-min observational period was recorded.

Functional immunohistochemistry

Ninety minutes following injection, the rats were deeply anaesthetized with sodium pentobarbitone ($100 \text{ mg}\cdot\text{kg}^{-1}$; B. Braun, Sheffield, UK) and perfused transcardially with heparinized ($10\,000 \text{ i.u.}\cdot\text{L}^{-1}$; Leo Pharma, Ballerup, Denmark) isotonic saline (0.9% NaCl) for 8 min, followed by 4% paraformaldehyde in 0.1 M phosphate buffer ($\text{pH } 7.0$) 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 phosphate buffer. Using a freezing-sledge microtome, $30 \mu\text{m}$ coronal sections throughout the rostro-caudal extent of the brain were cut. 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 phosphate buffer for 30 min at room temperature, followed by washes in 0.1 M phosphate buffer. To block non-specific staining, slices were incubated for 1 h at room temperature in 2% normal goat serum (2% NGS, 0.1 M phosphate buffer, 0.3% Triton X-100). Slices were then incubated for $\sim 24 \text{ h}$ at 4°C in goat anti-rabbit c-Fos antibody (SC052, Santa Cruz Biotechnology Inc., Santa Cruz, USA) diluted $1:1000$ in the NGS blocking serum, then sequentially in goat anti-rabbit immunoglobulin-peroxidase complex (Vector Laboratories, Peterborough, UK) diluted $1:500$ in blocking serum followed by streptavidin-biotin-horseradish peroxidase complex diluted $1:400$ in 0.1 M phosphate buffer. Staining was visualized using nickel-intensified diaminobenzidine (Vector Laboratories). 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 animal (4–8 sections) with the observer unaware of the treatment group. The average number of cells per section was calculated for each

animal and further averaged to provide a treatment group mean.

Extracellular electrophysiology

Rats were killed by cervical dislocation and decapitation under isoflurane anaesthesia. The brain was rapidly removed and dissected to form a tissue block containing the hypothalamus. Coronal brain slices ($400 \mu\text{m}$ thick) were cut in ice-cold, artificial cerebrospinal fluid (aCSF; 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 ; D-glucose, 5 mM ; adjusted to $\text{pH } 7.4$ and constantly perfused with $95\% \text{ O}_2/5\% \text{ CO}_2$) using a Vibroslicer (Campden Instruments, Loughborough, UK). Brain slices were transferred to a PDMI-2 submerged slice microincubator (Medical Systems Corp., New York, NY, USA) and maintained for 8–12 h by perfusion (approximately $1.5 \text{ mL}\cdot\text{min}^{-1}$) 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. Single-unit activity of hypothalamic neurones was recorded extracellularly with borosilicate glass electrodes (Harvard Instruments, Herts, UK) filled with 2 M NaCl (resistance approximately $5 \text{ M}\Omega$). Action potential spikes were amplified ($\times 20\,000$) and filtered (bandwidth 300 Hz to 3 kHz). Amplification, filtering and spike discrimination were performed using a NeuroLog modular system (Digitimer Ltd., Welwyn Garden City, UK). Data were collected and plotted as integrated histograms using Spike 2 software (Cambridge Electronic Design, Cambridge, UK). The size of the spikes recorded was between 10 and 100 mV . Only spikes at least two times the size of the background noise were recorded and the cell firing rate was recorded for at least 10 min to obtain a stable baseline firing rate, prior to a change in aCSF or drug treatment. 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 previous 5 min baseline recording.

Data analysis. Data on food intake are presented as means $\pm$ SEM, and were analysed using an unpaired *t*-test or by one-way ANOVA with *post hoc* Tukey's multiple comparison tests. We also present the total time spent in each behaviour during the BSS in Supporting Information Table S1, and analysed this using a non-parametric Kruskal–Wallis test with Dunn's *post hoc* comparisons. Results from the c-Fos immunohistochemistry were derived from each region of interest and means ($\pm$ SEM) from each region were compared independently using a Mann–Whitney *U*-test. Electrophysiological responses in VMN neurones are shown as mean firing rates ($\pm$ SEM) and compared with an unpaired *t*-test. In all experiments, significance was taken at $P < 0.05$.

Materials

All chemicals (including histamine dihydrochloride, thiopramide maleate, imetit dihydrobromide, pyrilamine maleate) were supplied by Sigma-Aldrich Company Ltd. (Poole, UK), unless stated otherwise. Proxyfan oxalate was bought from Tocris Bioscience (Bristol, UK).

Results

Compounds acting on histamine receptors affect feeding without disrupting other aspects of normal behaviour

Although the effect was relatively transient and food intake had normalized by 24 h, 200 nM histamine i.c.v. potentially decreased fast-induced re-feeding (Figure 1). The effect of centrally administered histamine was compared with that of two highly selective H_3 receptor ligands: the agonist, imetit, which has a K_i of 0.1–1 nM and the inverse agonist, thioperamide, which has a K_i of 1–5 nM at H_3 receptors, both with negligible affinity for other histamine and non-histamine receptors (Arrang *et al.*, 1987; Garbarg *et al.*, 1992). Imetit caused a dose-dependent increase in food intake in the first hour following injection when administered i.c.v. (Figure 2). The anorexigenic effect of thioperamide though noticeable earlier, only became significant statistically at the 4 h measurement when injected centrally. The two H_3 receptor-selective drugs were also effective when administered i.p., suggesting that they crossed the blood-brain barrier to have their action on feeding (Figure 2). Respectively, imetit (10 mg·kg⁻¹) and thioperamide (2 mg·kg⁻¹) produced strong orexigenic and anorexigenic effects, and were chosen to examine the behavioural satiety sequence more closely.

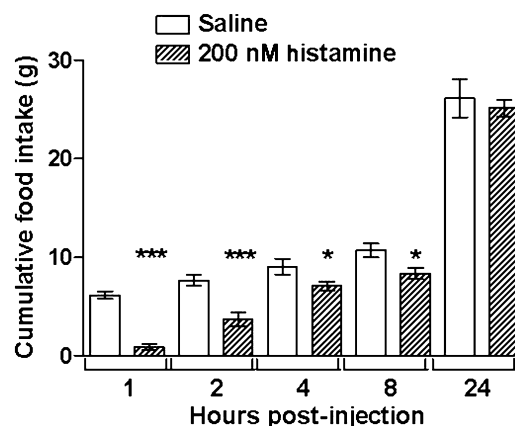


Figure 1

Food consumption measured at 1, 2, 4, 8 and 24 h after i.c.v. injection of histamine (200 nM, $n = 11$ per group) or control saline ($n = 9$). * $P < 0.05$, *** $P < 0.005$, significantly different from values with saline; unpaired t -test.

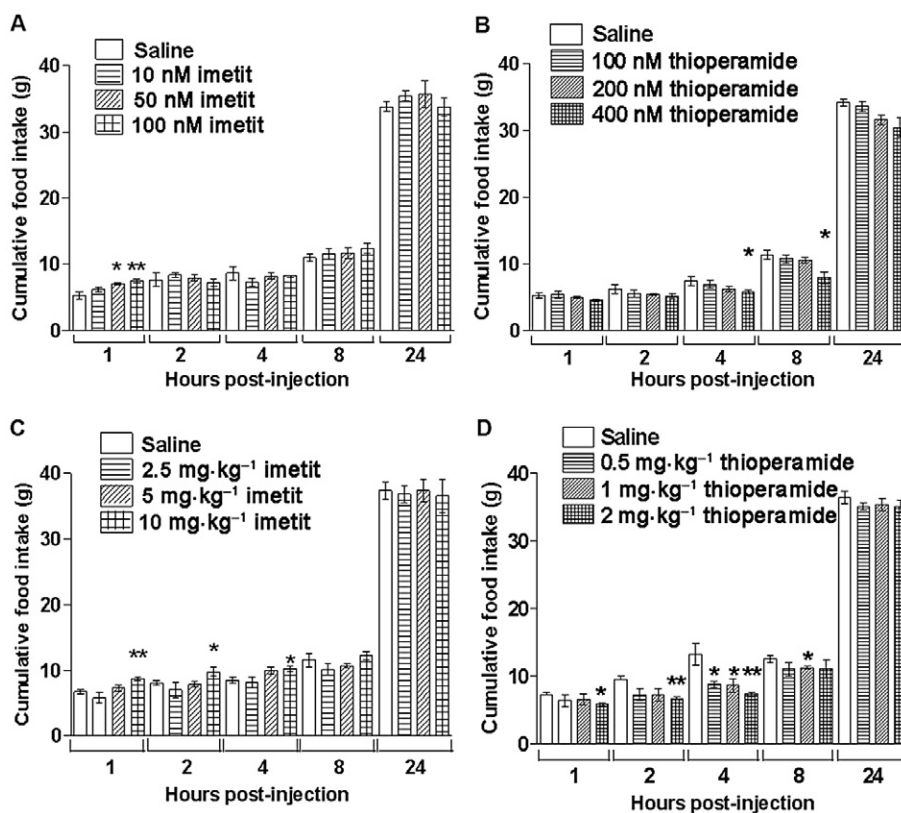


Figure 2

Food consumption measured at 1, 2, 4, 8 and 24 h after i.c.v. injection with (A) imetit (0, 10, 50 and 100 nM; $n = 6$ –7 per group) or (B) thioperamide (0, 100, 200 and 400 nM, $n = 5$). In separate experiments, food consumption was measured after i.p. injection with (C) imetit (0, 2.5, 5 and 10 mg·kg⁻¹; $n = 6$ –7) or (D) thioperamide (0, 0.5, 1 and 2 mg·kg⁻¹; $n = 5$). * $P < 0.05$, ** $P < 0.01$, significantly different from corresponding values with saline; one-way ANOVA with Tukey's multiple comparison *post hoc* test.

In the next experiment, each rat received three treatments with at least 3 days in between. Injections were given i.p. just after lights out, and normal night-time feeding behaviour recorded for the subsequent 90 min. Over this period, the drugs had the expected effects on food intake (average amount of chow eaten over 90 min: vehicle 6.01 g, imetit 7.24 g, thioperamide 4.39 g, both $P < 0.05$ compared with vehicle-treated group). The overall pattern of the behavioural satiety sequence was not disrupted by either treatment, suggesting the drugs were not having any adverse effects, such as causing nausea or sedation (Figure 3). Rats injected with imetit spent $34 \pm 3\%$ of their time eating, whereas control animals spent $31 \pm 3\%$ (not significant; Supporting Information Table S1). All times spent in other behaviours corresponded with those of control animals. Thioperamide-treated rats also showed behaviours similar to those of control rats 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 $24 \pm 3\%$ of their time feeding over the 90 min they were observed, although this did not quite reach statistical significance (Supporting Information Table S1).

Yoshimoto *et al.* (2006) found that in mice, thioperamide ($3\text{--}30\text{ mg}\cdot\text{kg}^{-1}$) given orally caused an increase in food intake which, although by a different route of administration, might suggest a species difference. Thus, we examined the effect of thioperamide ($10\text{--}20\text{ mg}\cdot\text{kg}^{-1}$, i.p.) in mice at lights off. Thioperamide caused a dose-dependent decrease in night-time food intake (vehicle 0.75 g; $10\text{ mg}\cdot\text{kg}^{-1}$ thioperamide 0.51 g, $P < 0.001$; $20\text{ mg}\cdot\text{kg}^{-1}$ thioperamide 0.27 g, $P < 0.01$; compared with vehicle group) in adult, outbred mice.

Anorectic doses of histamine and thioperamide activate hypothalamic nuclei

Thioperamide enters the brain, where most H_3 receptors are located, to reduce food intake. In order to determine whether thioperamide was having widespread effects in the brain, we compared the ability of anorectic doses of i.c.v. histamine and i.p. thioperamide to induce expression of the functional activity marker, c-Fos. Histamine (200 nM, i.c.v.) did not cause global activation of the brain, but instead, activity was distributed within clearly identifiable regions (Figure 4). The staining pattern of c-Fos was similar following administration of thioperamide ($2\text{ mg}\cdot\text{kg}^{-1}$, i.p.), and therefore, we focused on these regions of interest in our quantitative analyses. The regions of interest were the VMN, dorsomedial nucleus (DMN), arcuate nucleus (ARC) and paraventricular nucleus (PVN) in the hypothalamus, anterior hypothalamic area, lateral hypothalamic area (LHA), medial amygdala, anterior amygdala, central amygdala, tuberomammillary nucleus and the dorsal raphe nucleus. These regions of interest were counted, and most regions displayed a significant increase in the number of c-Fos-positive cells per section following treatment with histamine (Figure 5) or thioperamide (Supporting Information Figure S1). Although values for the VMN cover this whole anatomical nucleus, it is clear that activation is mainly in the dorsomedial part of the VMN, the region linked closest with the regulation of feeding (Hawke *et al.*, 2009). Therefore, we concentrated on this specific region when making electrophysiological recordings.

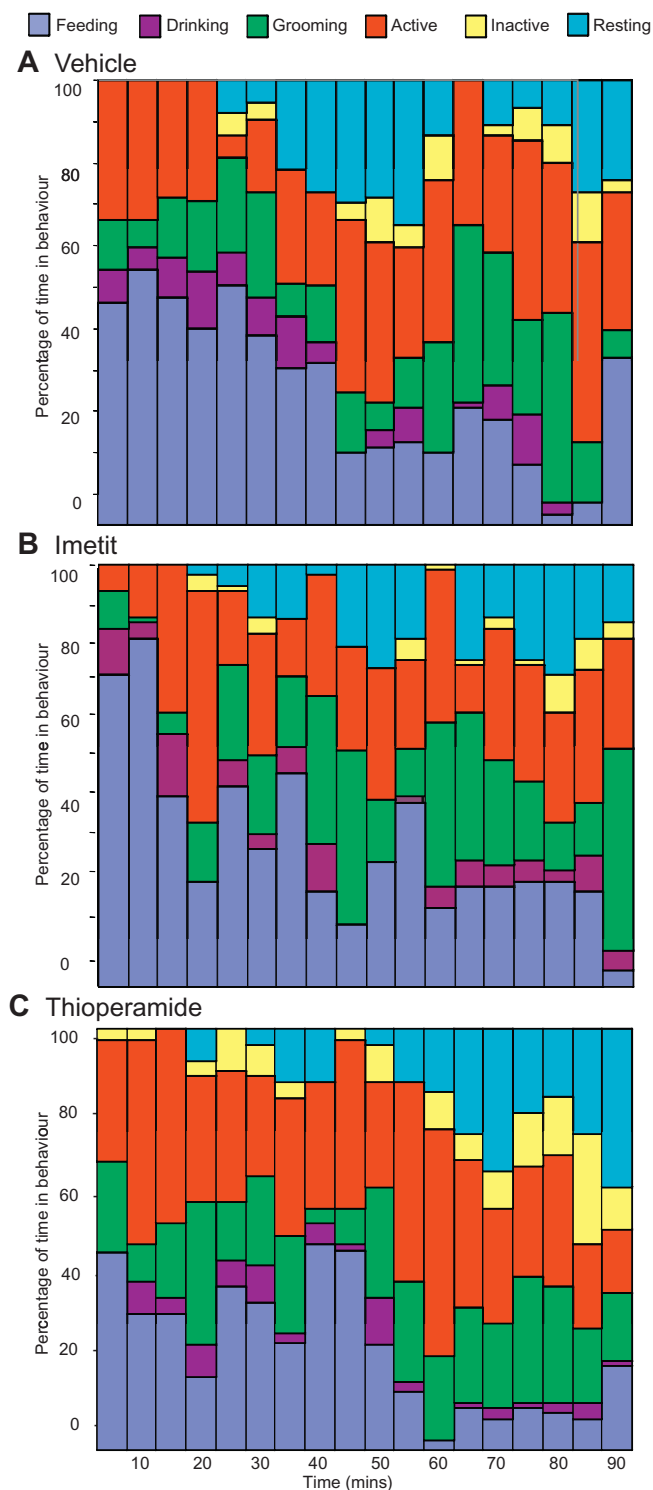


Figure 3

Behavioural satiety sequence. Rats ($n = 8$) were injected i.p. with (A) control saline, (B) imetit or (C) thioperamide, and introduced to food. Their behaviours were scored every 30 s for a period of 90 min according to the categories mentioned in the method section. Data were collated into 5 min time bins and expressed as mean percentage of total behaviour for each animal and then for the treatment. A comparison of percentage time spent in each behaviour over the whole 90 min period is shown in Supporting Information Table S1.

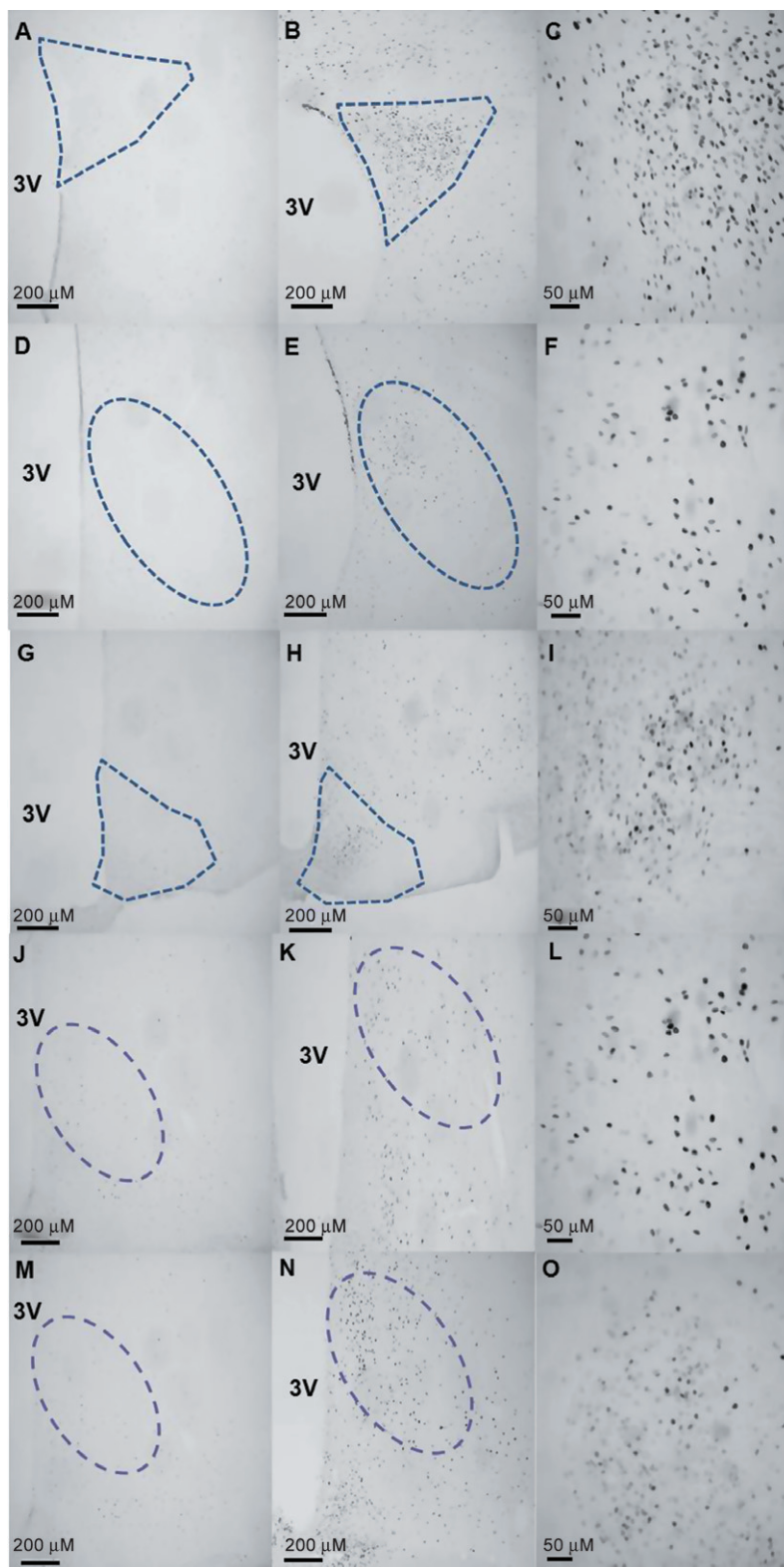


Figure 4

Representative photomicrographs of examples of regions of interest shown to exhibit a significantly high level of activation following histamine administration. Rats received an injection of control saline (A, D, G, J, M), histamine (200 nM; B, E, H), imetit (10 mg·kg⁻¹; K) or thioperamide (2 mg·kg⁻¹; N) into the cerebral ventricle 90 min before transcardial perfusion with fixative. Histamine is seen to induce the expression of c-Fos in the paraventricular (B, C), ventromedial (E, F) and arcuate (H, I) nuclei of the hypothalamus. Also shown, is c-Fos expression in the ventromedial nucleus following administration of imetit (K, L) or thioperamide (N, O).

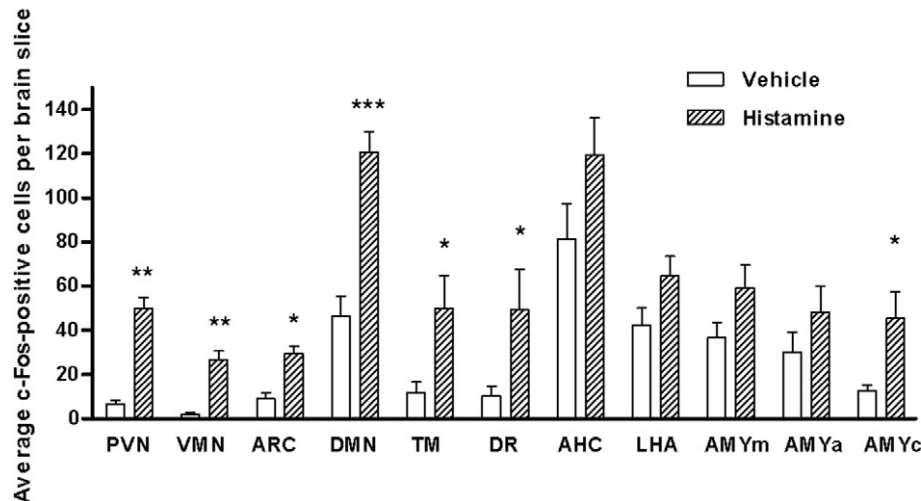


Figure 5

Quantification of the induction of c-Fos in regions of interest following administration of vehicle or 200 nM histamine (i.c.v.). The bars represent means and SEM of the number of Fos-positive nuclei per section. Four to eight sections were counted for each region in each animal. PVN, paraventricular nucleus; VMN, ventromedial nucleus; ARC, arcuate nucleus; DMN, dorsomedial nucleus; TM, tuberomammillary nucleus; DR, dorsal raphe nucleus; AHC, anterior hypothalamic area; LHA, lateral hypothalamic area; AMYm, medial amygdala; AMYa, anterior amygdala; AMYc, central amygdala. * $P < 0.05$; ** $P = 0.01$; *** $P = 0.001$, significantly different from values with vehicle; Mann–Whitney *U*-test.

Electrophysiological interactions between histamine H_3 and H_1 receptors in the dorsomedial VMN

A total of 197 spontaneously firing neurones in the dorsomedial region of the VMN were tested for their responsiveness to bath application of histamine (5 μ M). The cells showed an average basal firing rate of 1.91 ± 1.73 Hz and a range of 0.97–3.55 Hz. 122 out of 197 cells (62%) showed an increased firing when histamine was applied, whilst four decreased firing (2%) and 71 had no response (36%). Those that increased firing had an average rate of 3.47 ± 2.01 Hz following application of histamine. It is important to note that extracellular electrophysiology is biased towards recording from spontaneously active cells. Thus, the proportion of cells in each category is only an estimate, and more silent cells may be recruited by histamine than this estimation predicts. Thirty-one VMN neurones that were responsive to the application of histamine were then tested by the co-application of histamine (5 μ M) and pyrilamine (50 μ M), an H_1 receptor antagonist. In the majority of cells, pyrilamine blocked the effects of histamine (28 out of 31 cells, 90%; Figure 6A). All changes in neuronal activity were shown to return to basal levels within 20 min after application of either drug.

Forty-nine histamine-responsive VMN neurones were tested for their reaction to the application of the H_3 receptor inverse agonist, thioperamide (20 μ M) alone. Forty-three out of 49 cells (88%) showed an increase in firing when thioperamide was applied (average firing rate of 3.15 ± 2.01 Hz), whilst one cell decreased firing and five had no response. Further, we tested 15 histamine-responsive VMN neurones with 20 μ M thioperamide applied alone and then co-applied with 50 μ M pyrilamine. All 15 cells showed increases in neuronal firing with thioperamide alone, but this was blocked when pyrilamine was co-applied with the thioperamide (Figure 6B).

Twenty spontaneous (average basal firing rate of 1.86 ± 1.33 Hz), histamine-responsive VMN neurones were tested, but none responded to the application of 20 μ M proxyfan with a change in firing rate. However, this same dose of proxyfan was able to block the excitatory effects of thioperamide (Figure 6C). A further 22 cells were tested with the H_3 receptor agonist, imetit, alone. Of these, 19 (86%) showed a reduction in firing rate when imetit (5 μ M) was applied (average firing rate reduced from 1.86 ± 1.33 Hz to 0.47 ± 0.42 Hz). In 100% of cells tested (20/20), proxyfan blocked the inhibitory effect of imetit on firing (Figure 6D).

Proxyfan antagonizes the actions of both thioperamide and imetit on feeding

Proxyfan is capable of a spectrum of potencies depending on the level of spontaneous receptor activity. We had shown that, in our *in vitro* model, proxyfan acts as a neutral antagonist, so we now tested whether this was true also within an *in vivo* model of feeding. 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 of proxyfan alone at both lights out and at lights on, in rats that were fed *ad libitum*, ensuring a spectrum of endogenous histamine concentrations would be tested. Proxyfan injected i.p. in a range of doses (0.2–5.0 mg·kg⁻¹ body weight) had no effect on food intake in normally satiated animals during the day time, or in night-time feeding animals (Supporting Information Figure S2).

In two separate experiments using normal feeding rats during the night time, proxyfan (5 mg·kg⁻¹) blocked the orexigenic action of the H_3 receptor agonist, imetit (10 mg·kg⁻¹, i.p.) and the anorectic action of the H_3 receptor inverse agonist, thioperamide (2 mg·kg⁻¹, i.p.; Figure 7).

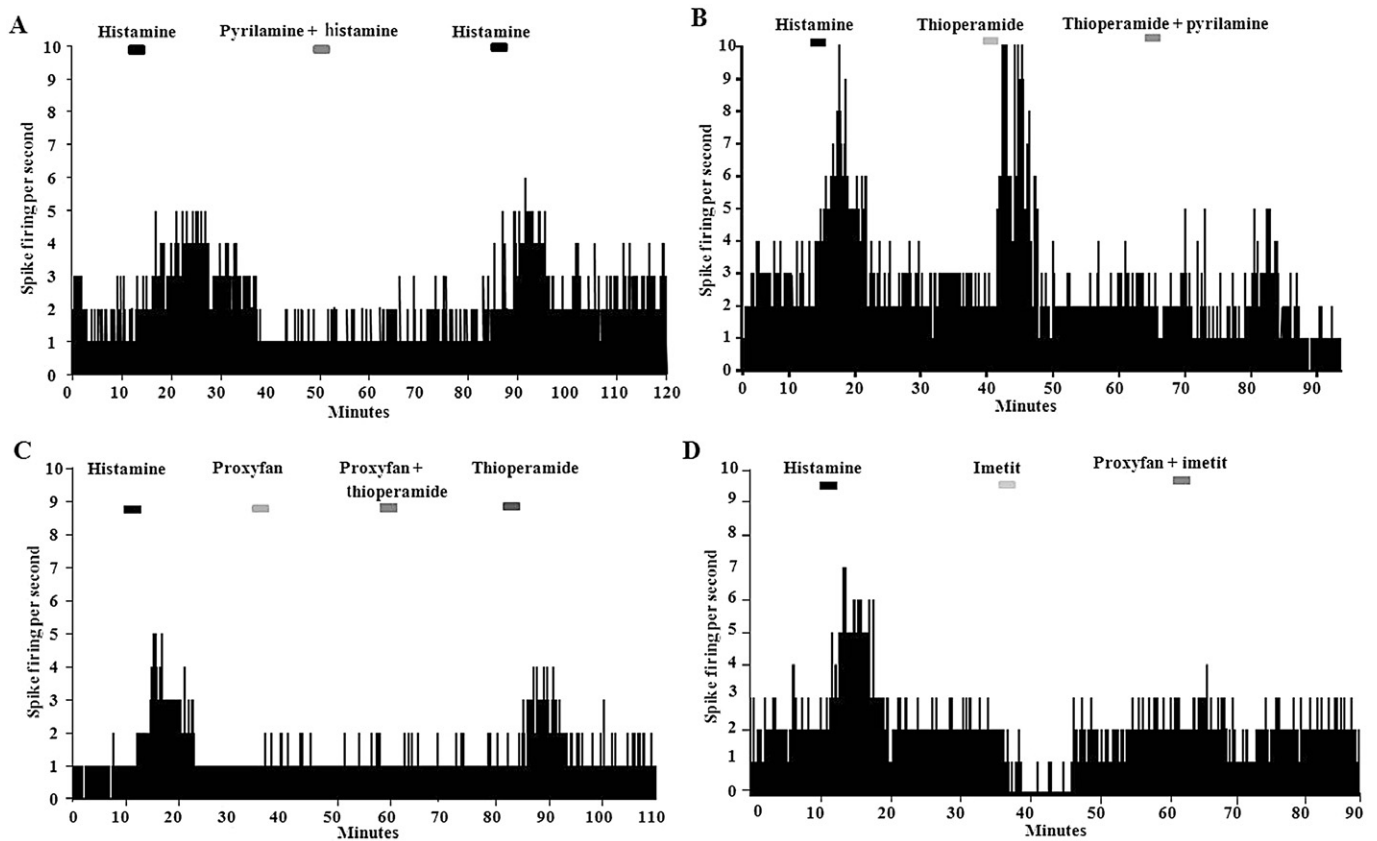


Figure 6

(A) Trace showing typical responses of a VMN neurone to the application of 5 μ M histamine alone or with co-administration of pyrilamine (an H_1 receptor antagonist; 50 μ M) using extracellular electrophysiology in a rat brain slice. Blocks at the top of the diagram indicate when the treatments were perfused in the bath (5 min applications). The Y-axis represents the firing rate of the neurone (spikes per second). A stable baseline was re-established between drug administrations. (B) Recording from another histamine-responsive neurone, showing increased firing when thioperamide (20 μ M) is applied. The effect of thioperamide is also blocked by pyrilamine. (C) VMN neurones do not respond to proxyfan when administered alone (20 μ M). However, proxyfan can block the activation caused by thioperamide. (D) 20 μ M imetit (20 μ M), the H_3 receptor agonist, causes an inhibition of histamine-responsive VMN neurones. This effect is also antagonized by proxyfan.

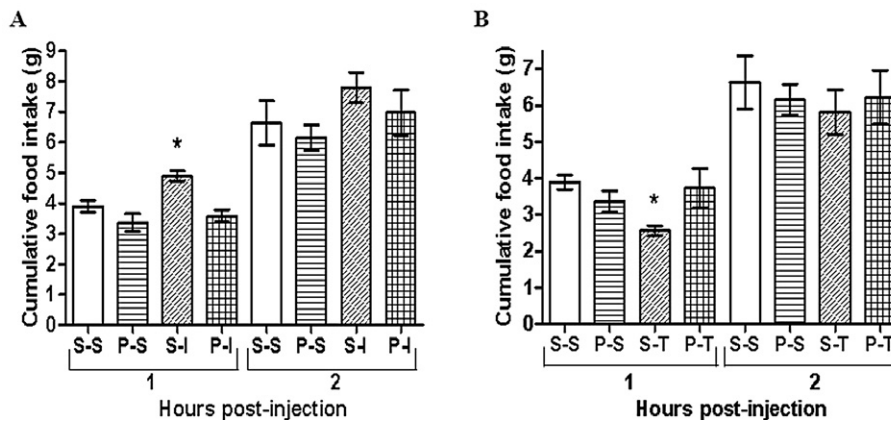


Figure 7

(A) Cumulative food consumption measured at 1 and 2 h after two i.p. injections with control saline (S-S), proxyfan (5 $\text{mg}\cdot\text{kg}^{-1}$) followed by saline (P-S), saline followed by imetit (10 $\text{mg}\cdot\text{kg}^{-1}$; S-I), or proxyfan followed by imetit (P-I). (B) In a separate experiment, injections were saline-saline (S-S), proxyfan-saline (P-S), saline-thioperamide (2 $\text{mg}\cdot\text{kg}^{-1}$; S-T), proxyfan-thioperamide (P-T). $n = 6$ in all groups; the two injections were given 15 min apart. $P < 0.05$; one-way ANOVA with Tukey's multiple comparison *post hoc* test.

Discussion

The effect of histamine in the CNS is anorexigenic and, therefore, overcoming the activity of presynaptic H₃ autoreceptors is hypothesized to increase the release of endogenous histamine and to reduce food intake (Hancock and Brune, 2005; Leurs *et al.*, 2005). However, three papers cast doubt on this theory. Firstly, Takahashi *et al.* (2002) reported that the H₃ receptor knockout mouse is mildly obese, which opposes what might be expected in the simplest model of a single population of receptor being involved in body-weight regulation. Secondly, in a study of dispersed neurones from the mediobasal hypothalamus, there was an H₃ receptor-mediated effect of histamine on GABA-dependent inhibitory postsynaptic potentials (Jang *et al.*, 2001). This would suggest that the H₃ receptors in this region may be heteroreceptors rather than presynaptic autoreceptors. Thirdly, Yoshimoto *et al.* (2006) suggested opposite effects of compounds acting on H₃ receptors when given orally, to those characterized in the main body of literature (Kent *et al.*, 1997; Itoh *et al.*, 1998; Lecklin and Tuomisto, 1998; Lecklin *et al.*, 1998; Hancock *et al.*, 2004; 2005; Malmlof *et al.*, 2005; 2006; 2007; Chiba *et al.*, 2009; Jethwa *et al.*, 2009).

Interpreting the phenotype of a receptor knockout animal in isolation can be problematic, and the authors who report that the H₃R knockout mice are only mildly obese, do point out that this may be due to up-regulation of other receptor systems in the mouse during development (Takahashi *et al.*, 2002). However, there are other explanations for the phenotype of the mouse which are dependent on the fact that histamine probably affects a wide variety of neurones, some of which might have opposing actions. For example, Fukagawa *et al.* (1989) reported that H₁ receptor antagonism decreased firing in 'glucose-excited' neurones of the VMN, but increased firing in 'glucose-inhibited' neurones of the LHA. Unfortunately, in this classic paper, the authors did not measure histaminergic actions on glucose-inhibited neurones in the VMN.

In our experiments, we have first shown that the anorectic effects of central histamine or the H₃ receptor inverse agonist, thioperamide, are associated with an increase in neuronal activity in the VMN, as measured in normally behaving animals. The behavioural satiety sequence was maintained when we administered thioperamide, suggesting that it reduces feeding without causing any adverse events, although we were unable to study all potential behavioural effects by this method. We then demonstrated *in vitro* that the excitatory effect of thioperamide on histamine-sensitive neurones in the VMN was blocked in each instance tested by co-administration of the H₁ receptor antagonist, pyrilamine. This is novel evidence that H₃ receptors are presynaptic autoreceptors located on histaminergic terminals in the VMN, tonically inhibiting the release of endogenous histamine. However, it is not absolute proof and, ideally, we would need to follow this up with patch-clamp recordings to confirm a reduction in histamine-mediated, excitatory postsynaptic potentials. Also, it does not rule out additional possible cellular locations of H₃ receptors. Jang *et al.* (2001) have used a preparation of dispersed hypothalamic neurones to demonstrate that H₃ receptor agonists can reduce inhibitory postsynaptic potentials. This would suggest H₃ receptors may

be present on inhibitory GABAergic terminals and might reduce the release of GABA locally. The VMN is surrounded by GABAergic neurones, which project into the VMN (King, 2006). However, a caveat of this work is that the dispersed neurones are probably from a large area of the mediobasal hypothalamus, rather than from the VMN alone (Jang *et al.*, 2001). The other detailed analysis of the effects of histamine on the electrical activity of VMN neurones comes from the laboratory of Pfaff and colleagues, who are interested in the arousing effects of histamine, and possible interactions with oestrogen, in the regulation of lordosis behaviour (Kow *et al.*, 2005; Zhou *et al.*, 2007; Dupre *et al.*, 2010). Their results show consistently a stimulatory effect of histamine through H₁ receptors, but very little effect through H₃ receptors. Despite the latter, they find strong expression of H₃ receptor message in individually harvested neurones, which could suggest a postsynaptic effect in the VMN. However, it is important to note that their recordings concentrate on the ventrolateral region of the VMN which has the strongest expression of oestrogen receptors and that this region is thought to be more functionally linked with female sexual behaviour rather than appetitive behaviour.

The results of Yoshimoto *et al.* (2006) are much more difficult to explain, as they found the opposite effect of compounds acting on H₃ receptors. For example, they report oral dosing with inverse agonist, thioperamide, increased food intake acutely and body weight over a period. Could there be relevant species differences between mice and rats? This seems unlikely, as there is previously published data showing that H₃ receptor inverse agonists reduce feeding in mice (Hancock *et al.*, 2004; 2005), rats (Itoh *et al.*, 1998; Lecklin and Tuomisto, 1998; Lecklin *et al.*, 1998; Hancock *et al.*, 2005; Malmlof *et al.*, 2005; 2006), hamsters (Jethwa *et al.*, 2009), pigs and primates (Malmlof *et al.*, 2007). Indeed, in a small experiment in the current paper, we too found a dose-dependent decrease in feeding with thioperamide. It is noteworthy also that, in a more recent publication from the Yoshimoto group, thioperamide inhibited neuropeptide Y- and nociceptin-induced hyperphagia (Yoshimoto *et al.*, 2008).

The original paper from Yoshimoto *et al.* (2006) found that the effects of H₃ receptor ligands were not apparent in H₃ receptor knockout mice. However, as stated previously, any knockout mouse may show compensatory developmental traits, so the use of a neutral antagonist might provide more valuable results. Proxyfan is reported to act as an antagonist at H₃ receptors, but it has the potential to act pharmacologically through a spectrum of potencies depending on the level of constitutive receptor activity (Gbahou *et al.*, 2003; Arrang *et al.*, 2007). In our *in vitro* electrophysiological preparation, we showed that, by itself, proxyfan had no effect on the electrical activity of VMN neurones, yet it could block the actions of both an H₃ receptor agonist and an inverse agonist. This suggests that in this model, proxyfan is acting as a neutral antagonist. Likewise, in both fed and fasted rats, proxyfan had no effect itself on feeding, but blocked the anorectic and orexigenic actions of thioperamide and imetit respectively. Thus, proxyfan appears to act as a neutral antagonist in *in vivo* models of feeding and, as previously published, drinking behaviours (Fox *et al.*, 2002). Thus, proxyfan will be a useful tool in the development of new drugs that target H₃ receptors involved in appetite and body-weight regulation.

Concerning the site and mode of action of compounds acting on H₃ receptors, we have concentrated on the dorso-medial region of the hypothalamic VMN. Functional activity marking with the immediate-early gene product, c-Fos, demonstrated the activation of this region by anorectic doses of both central histamine and systemic thioperamide. Although such methods will not determine whether the actions are direct, our subsequent electrophysiological recordings would suggest that this is the case, but would need to be proven absolutely by patch-clamp recordings. Other regions of the brain may be as important, and we noted clear activation also in the ARC, DMN and PVN of the hypothalamus. Interpretation is difficult as each of these areas is interconnected and might thus be driven transynaptically. Our electrophysiological data provide evidence for a direct effect at least in the VMN. Sakata *et al.* (1990) found that injections of a histamine synthesis inhibitor into either the VMN or the PVN elicited feeding, but it was ineffective in other hypothalamic regions. More recently, Barrett *et al.* (2005) have shown a season-dependent increase in electrical activity within the posterior dorsomedial region of the ARC in Siberian hamsters, which corresponds with a decrease in the expression of H₃ receptors in this area. They suggest a role for histamine and, in particular regulation of H₃ receptors, in seasonal control of body weight, but which might also be of important consequence to other species. Finally, Poole *et al.* (2008) found effects of histamine on neurones in the brainstem dorsal vagal complex, suggesting a potential modulatory role in brain-gut signalling. However, these powerful actions on individual neurones were not modified by H₃ receptor agonists. Furthermore, in our current experiments, we did not find an obvious effect of either thioperamide or imetit on the rate of satiation (the behavioural satiety sequence was maintained, but not shifted as might be expected for a satiety factor (Lawrence *et al.*, 2002; Scott *et al.*, 2005). Again, this fits with current hypotheses, that histamine is involved in the appetitive phase of feeding, rather than the consummatory phase (Passani *et al.*, 2011).

In conclusion, we provide strong pharmacological, anatomical and electrophysiological evidence that histamine has a major effect on neurones of the VMN, leading to a decrease in food intake. The effect is mediated by H₁ receptors on, as yet unidentified neurones in the dorsomedial region of the VMN and is modulated locally by H₃ receptors. This region contains the densest population of leptin-sensitive neurones in the VMN, which may contain the peptide, pituitary adenylate cyclase-activating polypeptide as a transmitter (Hawke *et al.*, 2009). Although there may be different locations of H₃ receptors, our evidence suggests that they are mainly presynaptic autoreceptors. Despite some recent controversies, H₃ receptors are still possible targets for the treatment of overeating, although it will be necessary to continue the development of selective and brain-penetrating drugs. This search will be facilitated with our finding that, in this system, proxyfan acts as a neutral antagonist both *in vitro* and *in vivo*.

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Conflicts of interest

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Quantification of the induction of c-Fos in regions of interest following administration of vehicle or thioperamide (2 mg·kg⁻¹, i.p.). The bars represent the number of Fos-positive nuclei per section (mean ± SEM). Groups were analysed using a Mann–Whitney *U*-test. **P* < 0.05; ***P* = 0.005. VMN, ventromedial nucleus; ARC, arcuate nucleus; DMN, dorsomedial nucleus; TM, tuberomammillary nucleus; DR, dorsal raphe nucleus; AHC, anterior hypothalamic area; AMYm, medial amygdala; AMYa, anterior amygdala; AMYc, central amygdala.

Figure S2 (A) Cumulative food consumption measured at 1, 2 and 4 h after i.p. injection with control saline vehicle (V) or increasing doses of proxyfan (0.2–5.0 mg·kg⁻¹). Data are from animals during the day time, when they are normally asleep. (B) In a separate experiment, injections were at night-time, when the animals are awake and feeding as normal. *n* = 6 in all groups. No significant effect of proxyfan by itself was recorded.

Table S1 The overall percentage of time spent in each behaviour over the 90-min observational period whilst carrying out the behavioural satiety sequence and after animals were injected with saline, imetit or thioperamide. Behaviours were categorized as follows: feeding, drinking, grooming active, inactive and resting. Data are means ± SEM. Groups were analysed using a Kruskal–Wallis test. Significant difference was seen in inactivity scores between thioperamide and imetit, but not the control, groups only. **P* < 0.05 (Dunn's *post hoc* test).

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