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Activation of the rat hypothalamic supramammillary nucleus by food anticipation, food restriction or ghrelin administration

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Authors' contributions

MVL, CH, NS, ES, TB and UB performed the research and analysed the data. CH, NS, JM and SLD designed the studies. MVL, CH, NS, JM and SLD wrote the paper.

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Abstract

The circulating orexigenic hormone ghrelin targets many brain areas involved in feeding control and signals via a dedicated receptor, the growth hormone secretagogue receptor 1A (GHSR-1A). One unexplored target area for ghrelin is the supramammillary nucleus (SuM), a hypothalamic area involved in motivation and reinforcement, and recently linked to metabolic control. Given that ghrelin binds to the SuM, we explored whether SuM cells respond to ghrelin and/or are activated when endogenous ghrelin levels are elevated. We found that peripheral ghrelin injection activates SuM cells in rats, reflected by an increase in the number of cells expressing c-Fos protein in this area and also by the predominantly excitatory response of single SuM cells recorded in *in vivo* electrophysiological studies. Further c-Fos mapping studies reveal that this area is also activated in rats in situations when circulating ghrelin levels are known to be elevated: in food-restricted rats anticipating the consumption of food and in fed rats anticipating the consumption of an energy-dense food. We also show that intra-SuM injection of ghrelin induces a feeding response in rats suggesting that, if peripheral ghrelin is able to access the SuM, it may have direct effects on this brain region. Collectively, our data demonstrate that the SuM is activated when peripheral ghrelin levels are high, further supporting the emerging role for this brain area in metabolic and feeding control.

Introduction

We now have a rather comprehensive, albeit incomplete, picture of the key brain pathways controlling energy balance and feeding behaviour. A great deal of this knowledge has been gathered by functional mapping of the target pathways engaged by circulating appetite-regulating hormones. Hypothalamic regions engaged in feeding control include the arcuate (ARC), ventromedial (VMH), supraoptic and paraventricular (PVN) nuclei. Other hypothalamic as well as midbrain regions, notably the lateral hypothalamus (LH), ventral tegmental area (VTA) and nucleus accumbens (NAcc), are involved in motivated behaviours for energy-dense foods that are high in fat and sugar (1). There is also emerging evidence for roles for forebrain regions associated with learning and emotion in feeding control including, for example, the ventral hippocampus (2).

Ghrelin is the only known circulating hormone that acts in the brain to increase food intake. It is powerfully orexigenic, inducing food-seeking (3), food anticipatory (4) and consummatory behaviours; directing what and how much is eaten and when (5, 6). The ghrelin receptor (the growth hormone secretagogue receptor 1A (GHSR-1A)) is expressed in brain regions important for energy homoestasis as well as those linked to reward, learning and memory (7). These include the hypothalamic ARC, PVN, LH, VMH and medial preoptic nuclei, the VTA, amygdala, hippocampus, NAcc and various brainstem areas including the nucleus of the solitary tract, the laterodorsal tegmental, dorsal raphe and parabrachial nuclei (7-10). Ghrelin appears to act at many levels throughout this integrated feeding network, involving direct and indirect effects at many of these sites.

Recently, the supramammillary nucleus (SuM) was identified as a site of potential relevance for metabolic control (11). The SuM is a midline region lying dorsally to the mammillary bodies and ventrally to the posterior hypothalamic area. The lateral SuM appears to have an important role in the generation and maintenance of hippocampal theta rhythm, while the medial SuM projects to areas involved in goal-oriented behaviours such as the lateral septum and LH (12). In addition, pharmacological manipulation of rat SuM neurones has revealed a potential role in motivated behaviour. Rats are motivated to self-administer a $GABA_A$ receptor antagonist into the SuM (13), and the presumed disinhibition of neural

activity after blockade of $GABA_A$ receptors results in c-Fos expression in the LH, VTA and NAcc (14). Excitation of the rat SuM via local injection of AMPA increases extracellular dopamine concentrations in the NAcc and induces a conditioned place preference (15). Furthermore, co-incident activation of the VTA and SuM is observed in conscious mice electrically self-stimulating the medial forebrain bundle (16). It is only recently that the SuM has been linked to feeding control, based on the findings that ghrelin binding sites are present in the SuM (17) and that an anorexigenic glucagon-like peptide 1 (GLP-1)-estrogen conjugate molecule can affect appetitive and consummatory behaviours at this site (11). The SuM has also been identified as neural substrate involved in other behaviours including aggression, sleep and arousal, circadian behaviours and responses to novelty. In the context of aggression, it has been suggested that the SuM may mediate reward-related aspects of this behaviour (18). In arousal, chemogenetic activation or inactivation of mouse SuMvglut2 neurons results in opposing effects on wakefulness (19). Similarly, in the rat, lesion of the SuM results in reduced c-Fos expression in the cortical areas active during REM sleep (20). In addition, c-Fos expression is increased in the SuM of rats placed in open field apparatus, pointing to a potential role in learning, novelty and/or stress (21). In the context of circadian behaviours, the rat SuM provides a dopaminergic input into the suprachiasmatic nucleus (SCN) and dopamine release follows a circadian pattern, with a peak at the onset of the rats' active period (22). Interestingly, these peaks are attenuated in rats made obese by a high-fat diet, pointing to a potential sensitivity in the SuM to metabolic/nutritional state and/or circulating appetite-related hormones.

Given the involvement of the SuM in behaviours relevant for feeding control, and its sensitivity to appetite-associated signals, we studied the influence of food as a naturally rewarding and reinforcing stimulus at this site. Using rats, we explored whether the neuronal activity of SuM cells (assessed by Fos mapping) is increased by ghrelin administration and also by physiological states in which endogenous ghrelin levels are known to be elevated: during anticipation of scheduled feeding and, in fed rats, when anticipating a chocolate treat (4, 23). In addition, we sought to determine whether i.v. ghrelin administration would alter/increase the electrical activity of SuM neurones recorded *in vivo*. Lastly, although it would be difficult to map the neural circuitry through which peripheral ghrelin engages the SuM, the fact that ghrelin binding

has been reported at this site (17), led us to explore the possibility that ghrelin has direct effects, assessed here by measuring the effect of intra-SuM ghrelin delivery on the feeding response.

Materials and methods

All procedures were approved by local and national ethical committees, in accordance with legal requirements of the European Commission. At the University of Edinburgh: under a UK Home Office project licence approved by the local ethics committee and in accordance with the UK Home Office Animals Scientific Procedures Act 1986. At the University of Gothenburg: Göteborgs djurförsöksetiska nämnd (ethical permit 45-2014).

All studies were performed on adult male Sprague-Dawley rats aged 8-10 weeks housed in a 12h light cycle (on at 07.00) at 20 ± 1°C and given *ad lib* access to water and a standard diet throughout (RM1; Special Diet Services, UK or Teklad Global 16% Protein Rodent Diet, Envigo, Madison, WI, USA) unless otherwise stated.

Experiment 1: c-Fos expression in the SuM of schedule-fed rats

To investigate the effect of food anticipation on c-Fos expression in the SuM during scheduled feeding (SF), we conditioned body weight-matched rats to scheduled access of a standard diet for seven days. During this time standard diet was available for 3 h during the light phase (either 13.00-16.00 or 14.00-17.00). It is well-established that rats can learn to anticipate periods of food access (24) and that this may involve ghrelin signalling (4). To discriminate anticipatory responses from responses to food, on day 8, we withheld standard diet ("SF-Unfed"), gave access at the expected time ("SF-Refed"), or gave access at an unexpected time ("SF-Unexp-Refed"; 4 h earlier than in the conditioning period; n = 8 in all groups). A control group (n = 8) had *ad lib* access to food and water throughout ("AL-Control").

On day 8, rats in the SF-Refed group were perfused-fixed 90 min after the beginning of the expected scheduled food access period (14.30 or 15.30) and the SF-Unexp-Refed group perfused-fixed 90 min after

the beginning of the unexpected food access period (10.30 or 11.30). Rats were administered with sodium pentobarbitone (200 mg/kg) i.p. and transcardially perfused with ice-cold 0.9% heparinised saline and 4% w/v paraformaldehyde in 0.1 M phosphate buffer (PB). Rats in the AL-Control group were perfused-fixed alongside the SF-Refed group, and rats in the SF-Unfed group were perfused-fixed at the normal time of scheduled feeding. Brains were removed, post-fixed in 4% w/v paraformaldehyde in PB containing 15% w/v sucrose, cryoprotected in PB containing 30% w/v sucrose, frozen on dry ice and cut serially on a freezing microtome at 44 µm in the coronal plane. Free-floating SuM containing sections were processed for c-Fos-like immunoreactivity using the DAB-hydrogen peroxidase method, where they were incubated with an anti-c-Fos rabbit primary antibody (Synaptic Systems, 226 003; 1:100,000), biotinylated horse anti-rabbit IgG secondary antibody (Vector Laboratories, BA-1100; 1:500), avidin-biotin complex (ABC; PK-6100, Vector Laboratories, VECTASTAIN Elite ABC Kit) and a DAB, nickel and hydrogen peroxide solution as previously described (25). Brain sections were mounted on gelatin-coated slides, dehydrated in increasing concentrations of alcohol (70% to 100%) followed by xylene (100%), coverslips applied with DPX mounting medium and imaged using a Leica DMR reflected light microscope. The SuM was identified with reference to a brain atlas (26). The number of SuM c-Fos positive nuclei was counted in a series of 4 brain sections between bregma -4.36 and -4.68 mm by an experienced experimenter under blinded conditions. The mean number of c-Fos-like immunoreactive (c-Fos+) nuclei per section was calculated for each brain and experimental group means calculated.

Experiment 2: c-Fos expression in the SuM in ad lib-fed rats schedule-fed sweetened condensed milk (SCM)

To determine the effect of scheduled access to an energy dense food on SuM c-Fos expression, we conditioned body weight-matched, *ad lib*-fed rats to scheduled SCM access for 15 min each day for 7 days. SCM (Nestlé, UK; diluted 50% v/v in water to 5 ml; 73 kJ) was presented in the rat's home cage in a heavy glass bowl during the light phase. All rats were habituated to an empty glass bowl for 48 h before SCM access.

Standard diet and water were available throughout scheduled SCM access. On day 8, one group received access at the expected scheduled time (13.00; "Exp-SCM", n = 8). As in Experiment 1, to discriminate effects of anticipation of SCM access on c-Fos expression from the effects of SCM consumption, a second group received access at an unexpected time: 4 h earlier than in the seven-day conditioning period ("Unexp-SCM", n = 8). To avoid environmental cues, on day 8 the Unexp-SCM group were given SCM in their home cage by an unfamiliar experimenter. A group that never received access to SCM served as the Control group. On day 8, the Exp-SCM group was perfused 1 hr following the end of the expected SCM access period (14.15) and the Unexp-SCM groups were perfused 1 hr following the end of the unexpected SCM access period (10.15). The Control group animals were perfused alongside the Exp-SCM group. For all groups, the brains were processed and analysed for c-Fos-like-immunoreactivity as described above. Rats that did not consume SCM on day 8 were excluded from the experiment.

Experiment 3: c-Fos expression in the SuM of rats fasted or given i.p. ghrelin

To investigate the effect of peripheral ghrelin injection on SuM c-Fos expression, body weight-matched rats were allocated into two groups; a "Vehicle" group which received an i.p. injection of saline (n = 5) and a "Ghrelin" group which received an i.p. injection of ghrelin (n = 7; 110 μg/kg; 1465, Tocris, Bristol, UK). The ghrelin dose used has previously been shown to induce a feeding response in rats[8]. The injections were balanced with respect to time of day for the two groups.

Ninety minutes after i.p. injection of ghrelin or vehicle, the rats were deeply anaesthetized with Rompun® vet (10 mg/kg; Bayer, Leverkusen, Germany) and Ketaminol® vet (75 mg/kg; Intervet, Boxmeer, Netherlands), perfused transcardially with heparinised 0.9% saline followed by 4% paraformaldehyde in 0.1M PB. The brains were post-fixed overnight at 4°C in 4% paraformaldehyde solution containing 15% sucrose and cryoprotected in 0.1M PB containing 30% sucrose at 4°C until cryosection. The brains were frozen and coronal sections (30 μm) containing the SuM were cut using a cryostat. Brain sections were processed for c-Fos immunohistochemistry where they were incubated with rabbit anti-c-Fos antibody

(1:20,000; Ab-5 (4-17) Rabbit pAb, PC38, Calbiochem) for two days at 4°C, followed by Alexa Fluor 488 goat anti-rabbit secondary antibody (1:250; IgG(H+L); A-11008, Invitrogen) for 1 h at room temperature before being mounted onto glass slides and coverslipped with ProLong® Diamond Antifade mountant (Life Technologies, Carlsbad, USA).

Images of the SuM containing sections were acquired using a Leica DMRB fluorescent microscope. The SuM was identified with reference to a rat brain atlas (26). The number of SuM c-Fos positive nuclei was counted in a series of 4 brain sections between bregma -4.36 to -4.68 mm under blinded conditions.

Experiment 4: In vivo *electrophysiology in the SuM of rats given i.v. ghrelin*

To investigate the effect of i.v. ghrelin injection on the electrical activity of SuM cells, single neurones were recorded from the SuM of rats (n = 34 with body weight ~ 350 g**;** 84 cells) under urethane anaesthesia (ethyl carbamate, 1.3 g/kg i.p.), using conventional extracellular recording techniques. A cannula was inserted into the femoral vein for i.v. injections. The rats were tracheotomised and the ventromedial surface of the hypothalamus at the level of the pituitary and neural stalk was exposed by transpharyngeal surgery. Under visual control, a glass microelectrode (tip diameter ~ 1 µm) filled with 1.5 % neurobiotin in 0.25 M NaCl (Vector Labs, Peterborough, UK) was placed < 1 mm rostral to the pituitary, on the midline, and lowered into the tissue by 2.5 mm from first contact with the arachnoid tissue. This consistently led the electrode tip into the SuM as established histologically by juxtacellular labelling of recorded cells in pilot experiments.

The mean spontaneous firing rate of SuM cells was recorded for at least 10 min in basal conditions then the cells were tested with i.v. injection of 10 µg ghrelin (Tocris; in 100 µl normal saline). Peripheral administration (i.v.) of this ghrelin dose has previously been shown to induce ARC c-Fos expression in rats[26]. In each cell tested, the firing rate in the 10 min before ghrelin injection was compared with the rate over a 20 min period starting 5 min after and ending 25 min after injection. We set a change of +0.5 spikes/s as an arbitrary threshold above which the cell was considered as activated by ghrelin, and a change of - 0.3

spikes/s as a threshold above which the cell was considered as inhibited**,** based on our previous study (27). Only cells that responded above those thresholds and with a significance of $p < 0.0001$ (comparison of mean (SD) firing rates in 30-s bins; Student's t test), were considered as significantly responsive. Within the subpopulation of SuM cells, the mean change in firing rate in the 20 min period starting 5 min after and ending 25 min after ghrelin injection was statistically tested to check whether, as a subpopulation response, this change is statistically different from 0. For this, we used a Wilcoxon Signed Rank test, accepting $P < 0.05$ as significant.

For all cells, interspike interval (ISI) histograms were constructed in 10 ms bins from at least 10 min of stationary spontaneous discharge activity (Spike 2 software; CED, Cambridge UK). Discharge was judged to be stationary if cells displayed a similar mean firing rate throughout the period of recording used for constructing the histogram. We then constructed hazard functions (28). These plot the incidence of spikes as a proportion of the size of the residual tail of the ISI distribution. When plotted this way, a negative exponential distribution (the distribution characteristic of random events) becomes a constant "hazard" proportional to the average firing rate. Deviation from this constant level is interpretable as periods of increased or decreased excitability. We calculated hazard functions in 10 ms bins by the formula (hazard in bin [t, t + 10]) = (number of ISIs in bin [t, t + 10]) / (number of ISIs of length > t). This calculates the hazard for each successive 10 ms bin. Hazard functions were normalised to the average hazard for that cell, and for subpopulations, consensus functions were calculated from the means of normalised hazard functions. The hazard function displays how the excitability of a cell changes with time since the last spike.

To confirm that recorded cells were located in the SuM, labelling of cells extracellularly-recorded *in vivo* was performed using the juxtacellular method first described by Pinault (29). After a SuM cell was tested with i.v. ghrelin and recorded long enough to be able to determine whether ghrelin induced a significant response or not, we applied anodal current pulses (200 ms, 50% duty cycle) in steps of increasing amplitude (1-10 nA) to eject neurobiotin from the electrode. Reliable labelling was obtained when the firing activity of the recorded neurone was robustly entrained by the positive current phase, and was kept so for 5-10 min. One neurone was labelled in each rat. At the end of the electrophysiology experiments, the rats were

euthanised with an overdose of sodium pentobarbitone and perfused. Brains were post-fixed, crypoprotected and sectioned coronally at 44 µm as described for Experiment 1. Floating sections were incubated with streptavidin conjugated to Alexa Fluor 594, and a Leica DMRB fluorescent microscope was used to visualise the presence of neurobiotin.

Experiment 5: Food intake after intra-SuM ghrelin administration

Male rats (250-310 g) were deeply anaesthetized by i.p. injection of a combination of Rompun® vet (10 mg/kg, Bayer) and Ketaminol® vet (75 mg/kg, Intervet) and placed in a stereotaxic frame. After exposure of the skull, small holes were drilled for anchoring screws and the guide cannula. A single 26 gauge guide cannula was inserted and fixed in place with dental cement. For placement of the guide cannula in the SuM, the following coordinates were used: AP: - 4.8 mm; ML: ± 0.7 mm; DV: - 6.5 mm with injector extending 2.5 mm below the tip of the guide cannula, resulting in a final depth of - 9.0 mm. After surgery, the wound was closed with stitches and each animal was injected subcutaneously with an analgesic (Rimadyl®, Orion Pharma Animal Health, Sollentuna, Sweden), singly housed and left to recover for at least 4 days.

Rats were habituated to handling/injecting on two occasions before the experimental days. Each animal was unilaterally injected into the SuM via pressure injection with two doses of ghrelin (1 µg or 0.5 µg, 0.3 µl per injection; 1465, Tocris) or vehicle (0.3 µl aCSF; Tocris) on different days, separated by a wash-out day, in a cross-over design so each animal was its own control (n= 13). The doses of ghrelin were based on a previously used intra-VTA dose known to induce a feeding response (30). All injections were performed early in the light phase. Food intake was measured at 3 h, 6 h and 24 h post-intra-SuM injection. At the end of the study, the animals were deeply anesthetized with isoflurane (Baxter, Deerfield, USA) and received an injection (0.3 µl) of Indian ink in the SuM before decapitation. Cannula placement was confirmed by visualising injected ink in frozen brain sections.

Statistical analysis

Where appropriate data are expressed as mean ± SEM. Statistical analysis was conducted using SPSS (version 25) or GraphPad Prism 6. Initially data were tested for normality using a Shapiro-Wilk test or a D'Agostino and Pearson omnibus normality test. For Experiment 1 (c-Fos expression with scheduled-feeding of chow), a one-way ANOVA with a Bonferroni post hoc test was used. For Experiment 2 (c-Fos expression with restricted SCM access), a Kruskal-Wallis test with a Dunn's multiple comparisons post hoc test was used. For Experiment 3 (c-Fos expression with i.p. ghrelin), a Mann Whitney U test was used. For Experiment 5 (food intake with intra-SuM ghrelin injection), a repeated measures one-way ANOVA with a Bonferroni post hoc test was used. Statistical significance was set at $p < 0.05$.

Results

Experiment 1: c-Fos expression in the SuM of schedule-fed rats

Compared to *ad lib*-fed controls, schedule-fed rats that were anticipating food access but were not refed had a significantly higher number of SuM c-Fos+ nuclei (AL-Control: 232.5 ± 44.1 c-Fos+ nuclei/section; SF-Unfed: 364.3 ± 20.7 c-Fos+ nuclei/section; $p = 0.025$; Figure 1). There was no significant difference in c-Fos expression between the SF-Unfed group and those schedule-fed at the expected time or at an unexpected time (SF-Refed: 394.5 ± 26.3 c-Fos+ nuclei/section; SF-Unexp-Refed: 383.9 ± 54.5 c-Fos+ nuclei/section; both $p > 0.99$).

Experiment 2: c-Fos expression in the SuM in ad lib*-fed rats schedule-fed SCM*

In *ad lib*-fed rats conditioned to receive daily access to SCM, rats consuming SCM at the expected time had a significantly higher number of SuM c-Fos+ nuclei than controls (Control: 55.1 ± 15.7 c-Fos+ nuclei/section; Exp-SCM: 199.1 ± 18.9 c-Fos+ nuclei/section; $p = 0.048$; Figure 2). Compared to controls, rats consuming

SCM at an unexpected time also had significantly higher number of SuM c-Fos+ nuclei (Unexp-SCM: 299.7 ± 23.0 c-Fos+ nuclei/section; p = 0.0005; Figure 2).

In a pilot study, we showed that in rats conditioned to receive SCM access, there was no significant difference in SuM c-Fos expression in rats that expected to receive SCM but had it withheld, compared to rats receiving and consuming SCM at the expected time (Expected-Withheld: 14.3 ± 3.0 c-Fos+ nuclei/section, n = 7; Expected-Received: 20.3 ± 4.0 c-Fos+ nuclei/section, n = 8; p = 0.23).

Experiment 3: c-Fos expression in the SuM of rats given i.p. ghrelin

Compared to vehicle-injected controls, rats injected with ghrelin had a significantly higher number of SuM c-Fos+ nuclei (Vehicle: 22.6 ± 6.0 c-Fos+ nuclei/section; Ghrelin: 61.2 ± 14.9 c-Fos+ nuclei/section; p = 0.018; Figure 3).

Experiment 4: In vivo *electrophysiology in the SuM of rats injected with i.v. ghrelin*

From 34 rats, we analysed the firing patterns of 84 spontaneously active neurones (mean rate 2.2 ± 0.27 spikes/s) recorded at depths consistent with localisation in the SuM. From their ISI distributions and hazard functions we classified these cells into three groups (Figure 4 & 5), as follows.

One group of 26 cells, that we call 'oscillatory cells', fired at 3.1 ± 0.54 spikes/s. These had multimodal ISI distributions, with one narrowly defined mode at < 20 ms, a second broader mode peaking at 270 ms in the summed distribution and a third mode peaking at 660 ms (the third mode was only apparent in cells that had a sufficiently large number of ISIs exceeding 400 ms; Figure 4A). These modes are apparent in the sum of the ISI distributions, which also shows a mode at 1040 ms (Figure 4B). This distribution of ISIs is similar to that described for oscillatory neurones in the VMH, and such distributions apparently arise from a sinusoidally oscillating synaptic input (27).

A second group of 27 cells, which we call 'broad' cells, fired at 2.3 ± 0.42 spikes/s. These had unimodal ISI distributions, with one broad mode peaking at about 140 ms in the sum of the ISI distributions (Figure 4A, & 4C). This distribution of ISIs is similar to that described for neurones in the VMH classified as broad cells, and interpreted as arising from a prolonged spike-dependent afterhyperpolarisation (27).

A third group of 31 cells, that we call 'doublet' cells (again following the terminology used for VMH cells[30]) fired at 2.3 ± 0.42 spikes/s. These had unimodal distributions with a single mode at < 30 ms (Figure 4A & 4D) and typically fired in intermittent clusters of 2-4 spikes (Figure 5A).

The summed ISI distributions overweight more active cells and mask neuronal heterogeneity. Figure 4B-D, therefore, shows the mean (SEM) ISI distributions of cells in each group, with each ISI distribution normalised to the total number of spikes in the distribution, and the corresponding mean (SEM) hazard functions, that display more directly the evolution of neuronal excitability following spikes.

We tested 39 SuM cells from 29 rats with i.v. ghrelin (10 μg). We tested the effects of ghrelin on up to two cells per rat, allowing at least 1 h between injections. In the cells responding to ghrelin, the response started between 2 to 5 min after ghrelin injection. We thus analysed the mean change in firing rate over the 20-min period taken between 5 min and 25 min after ghrelin injection (Figure 5D & 6).

Nine 'oscillatory' cells were tested, firing at an initial rate of 1.6 ± 0.5 spikes/s. The firing rate of 7 of these cells increased by > 0.5 spikes/s and that of one cell fell by 1.5 spikes/s. Overall the mean firing rate of the group increased by 1.1 ± 0.5 spikes/s (Figure 5D & 6C) but this increase was not significant (p = 0.07). Nine 'broad' cells were tested, firing at an initial rate of 2.5 ± 0.9 spikes/s. The firing rate of 7 of these cells increased by > 0.5 spikes/s, and the mean firing rate of this group increased significantly (p = 0.009) by 2.0 ± 0.6 spikes/s (Figure 5D & 6C). Thus, most of the 'broad' cells and most of the 'oscillatory' cells were activated by ghrelin, and their responses were delayed in onset and long lasting.

By contrast, only one of 14 'doublet' cells tested was activated by ghrelin. These cells fired at an initial mean rate of 1.0 ± 0.2 spikes/s, and the mean firing rate fell significantly (p = 0.025) after ghrelin by 0.17 ± 0.09 spikes/s. Another seven cells were tested that were firing too slowly (at 0.3 ± 0.1 spikes/s) to be

confidently classified; none of these were activated by ghrelin, and the mean rate of this group fell by 0.04 ± 0.04 spikes/s (Figure 5D & 6C).

Experiment 5: Food intake with intra-SuM ghrelin

In comparison to vehicle, intra-SuM injection of ghrelin increased food intake at 3 h post-injection for both doses (Vehicle: 1.6 ± 0.4 g; 0.5 μg Ghrelin: 3.5 ± 0.5 g; 1 μg Ghrelin: 4.6 ± 0.5 g; $p = 0.006$ and $p = 0.0006$ respectively; Figure 7B). Likewise, food intake at 6 h post-injection was also increased by both intra-SuM ghrelin doses (Vehicle: 3.0 ± 0.5 g; 0.5 μg Ghrelin: 4.9 ± 0.6 g; 1 μg Ghrelin: 5.9 ± 0.5 g; $p = 0.016$ and $p < 0.0001$ respectively; Figure 7C). There was no significant difference in food intake at 24 h post intra-SuM ghrelin injection for either dose (data not shown).

Discussion

This study identifies the SuM as a brain area that is activated when rats are hungry and/or anticipating food, and by peripheral administration of the orexigenic hormone, ghrelin. Ghrelin administered peripherally activates cells in this region as shown by *in vivo* electrophysiological studies and the detection of c-Fos-like immunoreactivity. The number of SuM cells expressing c-Fos-like immunoreactivity is also increased when rats are food restricted and anticipating food and when rats are satiated and anticipating an energy-dense food. Both of these physiological states are associated with increased ghrelin secretion (4, 23). Finally, we demonstrate that central ghrelin injection directed at the SuM drives a feeding response.

We carried out scheduled re-feeding of food-restricted rats, or gave satiated rats scheduled access to an energy-dense food. The aim was to determine whether metabolic state and access or anticipation of access to normal or palatable food, had an effect on c-Fos expression in the SuM. In our first experiment, food-restricted unfed rats showed a significant increase in the number of c-Fos+ cells in the SuM compared to *ad lib*-fed controls. Re-feeding had no effect on the number of c-Fos+ cells in the SuM compared to not

refeeding, regardless of whether rats were anticipating food access. Similarly, in our second experiment, *ad lib*-fed rats conditioned to receive regularly-scheduled access to SCM showed a significant increase in the number of c-Fos+ cells in the SuM compared to controls, regardless of whether the rats were anticipating SCM access. The increase in the number of SuM c-Fos+ cells was larger in rats receiving access to SCM at an unexpected time, but this difference was not significant compared to rats receiving access to SCM at the expected time. Nonetheless, these data may reflect the potential role of the SuM in appetite-related behaviour (11) and motivated behaviour (13, 14) where the unanticipated presentation of a familiar energy-dense food may trigger reinforced consummatory behaviour. Additionally, to mimic a physiological aspect of the hungry state, we administered ghrelin to satiated rats. Compared to vehicle injection, peripheral administration of ghrelin to satiated rats resulted in an increase in the number of c-Fos+ cells in the SuM.

Taken together, these data indicate that the number of c-Fos+ SuM cells is relatively low in the satiated state, but that the SuM is activated by peripheral ghrelin administration and after actual or anticipated food access. However, it is unclear whether the same population of SuM cells were c-Fos+ after food access or during anticipation of access. Likewise, it is not clear whether the same population of SuM cells were c-Fos+ in rats anticipating food and those administered ghrelin.

In general, the neuroanatomy of the SuM has not been well-defined in previous literature. However, some studies show that the SuM contains small to medium sized spherical shaped neurones, which are more densely packed in the medial SuM region compared to the lateral SuM region (31, 32). The SuM is bordered by multiple nuclei including the LH, perifornical nucleus, posterior hypothalamic area and VTA (31). Differences in cellular organisation between the SuM and bordering regions demonstrate that the SuM is a defined nucleus (31). Immunohistochemical studies have shown SuM neurones to express tyrosine hydroxylase, cholecystokinin (CCK), substance P and vasoactive intestinal peptide alongside other peptides (33). However, this pattern of expression is not specific to the SuM and it is not known whether a specific marker exists for SuM neurones.

The hormone sensitivity of the SuM is not well-characterised. Feeding control is regulated by a number of central orexigenic and anorexigenic signals. One such signal is oxytocin and we have previously shown that gavage of SCM activates magnocellular oxytocin cells (25). Oxytocin can be released into the brain from central dendrites to act at distant sites (34), oxytocin-immunoreactive nerve fibres are reported to enter the SuM (35), and oxytocin binding sites are present in the rat SuM (36). In acute brain slice preparations, oxytocin receptor agonists activate around half of SuM cells tested (35). It would be of interest to determine whether oxytocin signalling is relevant to the increase in c-Fos+ cells seen after food consumption, particularly SCM consumption.

Given ghrelin's well-established roles in food anticipation, the patterns of c-Fos expression seen in our c-Fos mapping studies point to the orexigenic hormone as a potential mediator of the effects observed in the SuM. To examine directly the effects of ghrelin on neuronal excitability in the SuM we used *in vivo* electrophysiology. We present here a preliminary characterisation of the *in vivo* firing patterns of rat SuM cells. Based on these patterns, we classified cells as oscillatory, broad or doublet cells. We found that systemic ghrelin injection was excitatory in 78% of the oscillatory cells. These cells may be of particular interest as they show a rhythmic firing pattern, consistent with an underlying sinusoidal oscillation in excitability at a frequency of about 4 Hz (i.e. in the low theta range), and there is evidence for a role for the SuM in driving rhythmic theta activity in the hippocampus. Hippocampal theta oscillations are associated with a range of cognitive and behavioural functions including learning, spatial and temporal memory, locomotion and emotion (37). SuM neurones project to the dentate gyrus and CA2 area of the hippocampus (38), and pharmacological blockade of the SuM in conscious or anaesthetised rats disrupts hippocampal theta rhythm (39). In urethane-anaesthetised rats, SuM neurones show at least four patterns of rhythmic activity, each has a phase-locked relationship, either in-or out-of-phase, with theta field activity recorded in the hippocampus (40). An *in vivo* electrophysiological study in the SuM and mammillary body of the urethane-anaesthetised rat studied the relationship between the electrical activity of single SuM cells and the hippocampal EEG (41). It was shown that 17% of cells fired synchronously with hippocampal theta. Taken together, this suggests that SuM neurones may generate or relay a rhythmic input that influences hippocampal theta oscillations.

The significance of theta rhythms in the brain remains conjectural. However, it has been noted that if a brain region “A” generating a theta rhythm projects to two areas “B” and “C” to induce a theta rhythm in these target sites, then an excitatory connection between B and C will be facilitated by the rhythmic coincidence of increased presynaptic excitability (in B) with increased postsynaptic excitability (in C) – thus facilitating functional connectivity between B and C. Such arguments suggest that theta rhythms may be of particular importance to information processing in the brain (42). In support of a role for the SuM in enhancing functional connectivity in the brain, it has been suggested that, for rats navigating a T-maze, information about the planned decision (encoded in the medial prefrontal cortex (mPFC)) is integrated with hippocampal spatial maps via a mPFC-thalamic-nucleus reuniens (NR)-hippocampal CA1 circuit (21). Just before the decision point, mPFC and NR neurones fire in coordination with hippocampal CA1 theta rhythm, and coordination with CA1 theta rhythm is also observed in the SuM. Optogenetic silencing of SuM neurones reduces temporal coordination in the mPFC-NR-CA1 circuit, as such the SuM may “gate” information flow in the mPFC-NR-CA1 circuit. In the context of the simple model described above, the A-to-B circuit could be considered as the mPFC-NR-CA1 circuit, and the SuM could be considered as population C. Given our identification of ghrelin-sensitive oscillatory cells in the SuM, it would be of interest to determine whether these cells are an important part of the brain’s learning and memory circuits, and to study the effects of appetite-related hormones on the functional connectivity of these circuits.

The electrophysiological approach used here requires that the recorded cells have some spontaneous activity at baseline, making it possible to observe inhibitory as well as excitatory responses, but inevitably is biased against recording neurones that have very little or no spontaneous activity. As reported for other ghrelin-responsive brain areas, such as the ARC (43), there is heterogeneity in the response of SuM cells. However, responses in the SuM were significant, long lasting, and comparable in magnitude to responses of ARC neurones to ghrelin receptor agonists *in vivo*, indicating that the SuM is a relevant ghrelin-responsive area of the hypothalamus (43).

Our demonstration that peripheral ghrelin injection both alters the electrical activity of SuM cells recorded in anaesthetised rats and increases the number of SuM cells expressing c-Fos provides compelling evidence

that the SuM is a relevant brain structure for ghrelin's neurobiological effects as it is also activated at times when endogenous ghrelin levels are high. Circulating ghrelin is known to access the brain. For instance, previous studies showed that peripheral ghrelin binds to the ARC (via the median eminence) and the hippocampus (44, 45). As centrally injected ghrelin binds to the SuM (17), the possibility exists that peripheral ghrelin could access the SuM and activate these cells directly.

Although difficult to map the neural circuitry engaged by peripheral ghrelin to cause this activation of SuM cells, the presence of ghrelin binding sites in this area raises the possibility that the SuM itself is part of this neurocircuitry. Ghrelin is powerfully orexigenic and the fact that intra-SuM ghrelin delivery was able to drive a feeding response suggests that ghrelin-responsive (GHSR-1A-expressing) cells in this area, when activated, can contribute to an orexigenic response. Activation of these cells would require either that peripheral ghrelin is able to reach the SuM or that other mechanisms exist to control the activity of this receptor, such a through heterodimerization with other receptors (46) or indeed by the recently discovered endogenous antagonist, leap2 (47). The fact that GHSR1A has constitutive activity (48) means that ghrelin-responsive pathways may not require ghrelin for their activation. To explore possible direct effects of ghrelin at the level of the SuM, we injected ghrelin directly at this site and were able to observe a feeding response. If peripheral ghrelin is able to access the SuM, it may be that those cells responsive to ghrelin in this area contribute to ghrelin's orexigenic effect. It would be important to determine how other ghrelin-responsive targets connect to the SuM for a deeper understanding of the neuronal network mediating ghrelin's behavioural effects. Indeed, these feeding data should be interpreted with some caution because, as for any intracranial injection, intra-SuM injected ghrelin might diffuse to neighbouring sites such as the LH and VTA, areas from which ghrelin can also drive a feeding response.

We showed that food anticipation also increased the number of SuM cells that express c-Fos, suggesting that the SuM might be a relevant brain area in the appetitive period of feeding. This increase in the number of c-Fos-expressing cells was seen regardless whether food was presented or absent at the expected time. This was previously seen in other hypothalamic nucleus important in feeding behaviours (ARC, LH, VMH and dorsomedial hypothalamus) (49).Moreover, given that circulating levels of ghrelin are correlated with

food anticipation and that central ghrelin injection increases behaviours associated with food anticipation (4, 23), the SuM might be activated, at least in part, by ghrelin during food anticipation. This contrasts, for example, with the AgRP neurones in the ARC that are activated when mice anticipate food but rapidly suppressed when food becomes available (50)

In summary, our data identify the SuM as a brain area with a potential role in metabolic control. Ghrelin may directly target the SuM for its neurobiological effects, although it is possible that it may also act indirectly via afferent pathways. Further studies are needed to explore the role of the SuM and its neuronal connections in ghrelin-regulated feeding behaviours, including the neurochemical identity of the cells and pathways engaged. The SuM is known to be involved in reward and memory (12, 15) as is ghrelin (3, 10, 30, 45). As such, further study of the SuM and its circuits may highlight the SuM as a region that integrates and combines information related to motivation, memory and food intake to induce complex feeding behaviours.

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Conflict of interests

The authors of the manuscript have no conflicts of interest to declare. **Figure legends**

Figure 1 – The effect of scheduled-feeding on SuM c-Fos expression. (A) Number of SuM nuclei (per brain section) expressing c-Fos after *ad libitum* access to bland diet (AL-Control; n = 8), bland diet scheduled-feeding when not refed (SF-Unfed; n = 8), bland diet scheduled-feeding when refed (SF-Refed; n = 8), or bland diet scheduled feeding at an unexpected time (SF-Unexp-Refed; n = 8). Data shown as mean ± SEM with individual data points plotted. One-way ANOVA ($F_{(3,28)} = 1.68$, $p = 0.022$) with Bonferroni (*$p < 0.05$). **(B)** Representative images of SuM c-Fos expression for all groups. Bregma -4.56 mm[24]. MM, medial mammillary nucleus; mp, mammillary peduncle.

Figure 2 – The effect of SCM scheduled-feeding on SuM c-Fos expression. (A) Number of SuM nuclei (per brain section) expressing c-Fos after *ad libitum* access to bland diet (Control; n = 8), SCM scheduled-feeding with SCM access at the scheduled time (Exp-SCM; n = 6) or SCM access prior to the scheduled time (Unexp-SCM; n = 5). Data shown as mean ± SEM with individual data points plotted. Kruskal-Wallis test (Kruskal-Wallis statistic = 15.16, $p < 0.0001$) with Dunn's multiple comparisons (*$p < 0.05$, ***$p < 0.001$). **(B)** Representative images of SuM c-Fos expression for all groups. Bregma -4.44 mm[24]. MM, medial mammillary nucleus; mp, mammillary peduncle.

Figure 3 – The effect of ghrelin on SuM c-Fos expression. (A) Number of SuM nuclei (per section) expressing c-Fos after i.p. vehicle (Vehicle; n = 5) and i.p. ghrelin (Ghrelin; n = 7). Data shown as mean ± SEM with individual data points plotted. Mann Whitney U test (U statistic = 3; *$p < 0.05$). **(B)** Representative images of SuM c-Fos expression for both groups. Bregma -4.56/4.68 mm[24]. MM, medial mammillary nucleus; mp, mammillary peduncle.

Figure 4 - Classification of SuM neurones recorded from *in vivo*. (A) Summed ISI distributions from 26 'oscillatory' cells (top), 27 'broad' cells (middle) and 31 'doublet' cells (bottom), plotted on a log scale. The 'oscillatory' cells display multimodal ISI distributions, with a mode at < 30 ms, and subsequent modes

(arrowed) at about 350 ms and 700 ms – a further mode at just over 1000s also appears in the summed distribution. The ‘broad’ cells show a single mode at 100-200 ms, and the ‘doublet’ cells a single mode at < 30 ms. **(B)** ‘oscillatory’ cells; **(C)** ‘broad’ cells; **(D)** ‘doublet’ cells. The graphs on the left show consensus ISI distributions (means ± SEM) in 10-ms bins. The graphs to the right show the corresponding hazard functions. The number of cells in each group is indicated by N. The hazard functions for broad cells are truncated at 0.3 s because for many cells in this group there were too few ISIs of > 300 ms to calculate meaningful hazards.

Figure 5 – Representative recordings for each SuM cell firing pattern *in vivo*. The left panels show an ‘oscillatory’ cell example, the middle panels a ‘broad’ cell example and the right panels a ‘doublet’ cell example. **(A)** An extract of the raw spike trace and an averaged spike waveform for each cell. **(B)** ISI distributions (in 10-ms bins) from 10 min of spontaneous activity for each cell. **(C)** Corresponding hazard functions for each cell. The hazard function of the broad cell is truncated to 0.3 s because hazard calculations become erratic when spike numbers in each bin are very low. **(D)** Response of each cell to i.v. ghrelin (arrow), shown as changes in firing rate from the average firing rate over the 5 min before injection.

Figure 6 - Responses of recorded SuM neurones to i.v. injections of ghrelin in neurones classified by firing pattern. (A) Response to ghrelin in all tested cells, ranked by response magnitude with ‘oscillatory’ cells in red, ‘doublet’ cells in green, ‘broad’ cells in orange and 7 slow (unclassified) cells in blue. Each bar represents one SuM neurone and its response averaged over the 20 min period taken between 5 min and 25 min after ghrelin injection. **(B)** Mean (± SEM) hazard functions of the 9 ‘oscillatory’ cells (left panel), the 9 ‘broad’ cells (middle panel), and the 14 ‘doublet’ cells (right panel). **(C)** Mean (± SEM) responses of these cells to i.v. ghrelin, calculated as the mean difference in firing rate (in 5-min bins) from the firing rate in the 5 min before injection.

Figure 7 - Effect of injection of ghrelin (0.5 µg and 1 µg doses) directed at the SuM on chow intake (g).

Data shown as mean ± SEM. n = 13. **(A)** Representative brain section showing SuM injection site (ink injection; right side) and corresponding rat brain atlas section (left side). Bregma -4.68mm[24]. **(B)** Cumulative chow intake at 3 h post intra-SuM ghrelin injection (repeated measures one-way ANOVA ($F_{(1.4,17.3)} = 21.19$, $p < 0.0001$) with Bonferroni (**p < 0.01, ***p < 0.001). **(C)** Cumulative chow intake at 6 h post intra-SuM ghrelin injection (repeated measures one-way ANOVA ($F_{(1.8,21.8)} = 15.96$, $p < 0.0001$) with Bonferroni (*p < 0.05, ****p < 0.0001).

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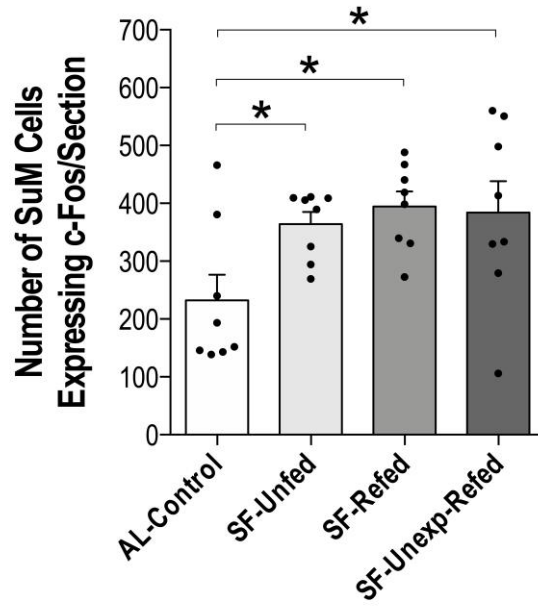
Figure 1



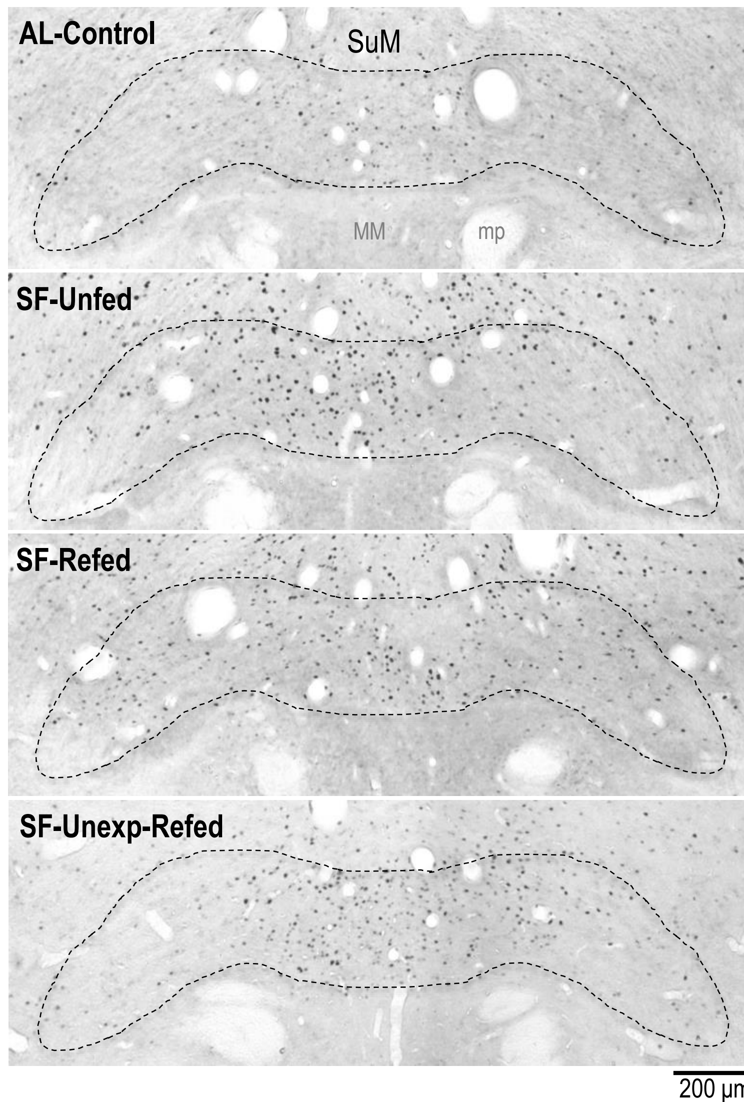
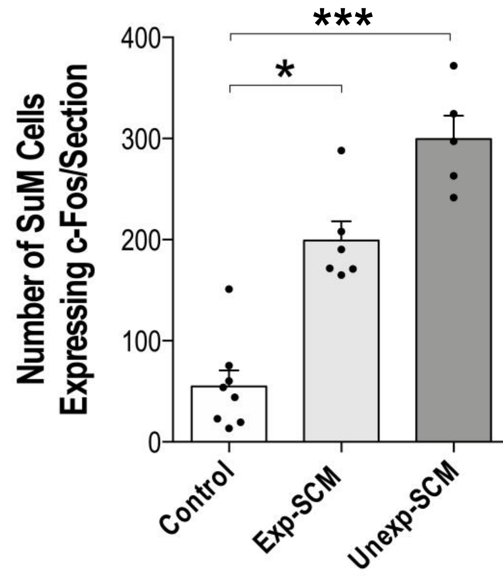


Figure 2



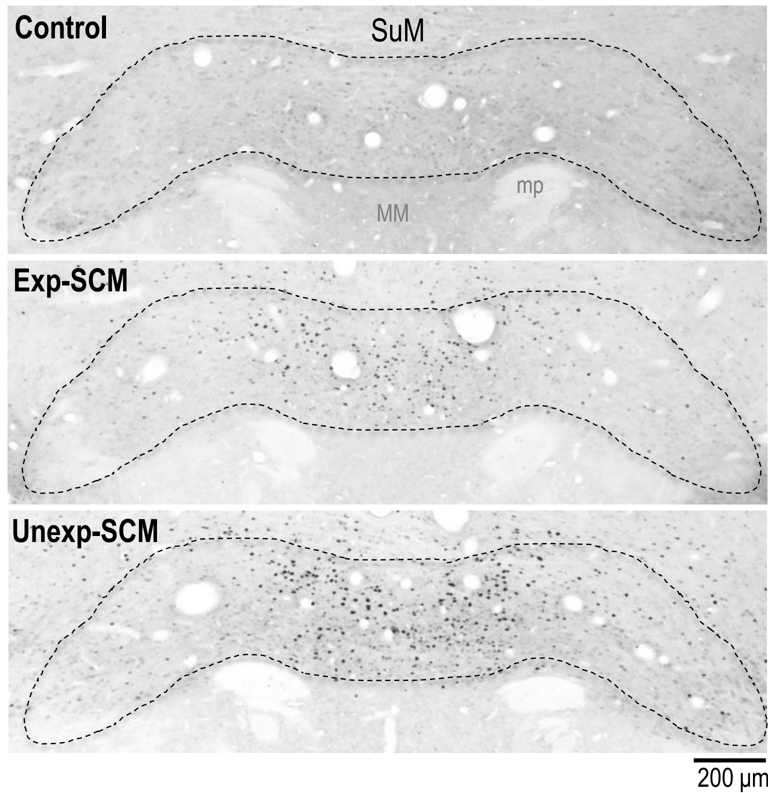
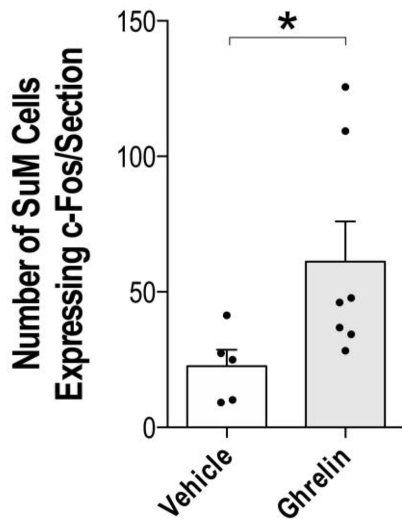


Figure 3

A



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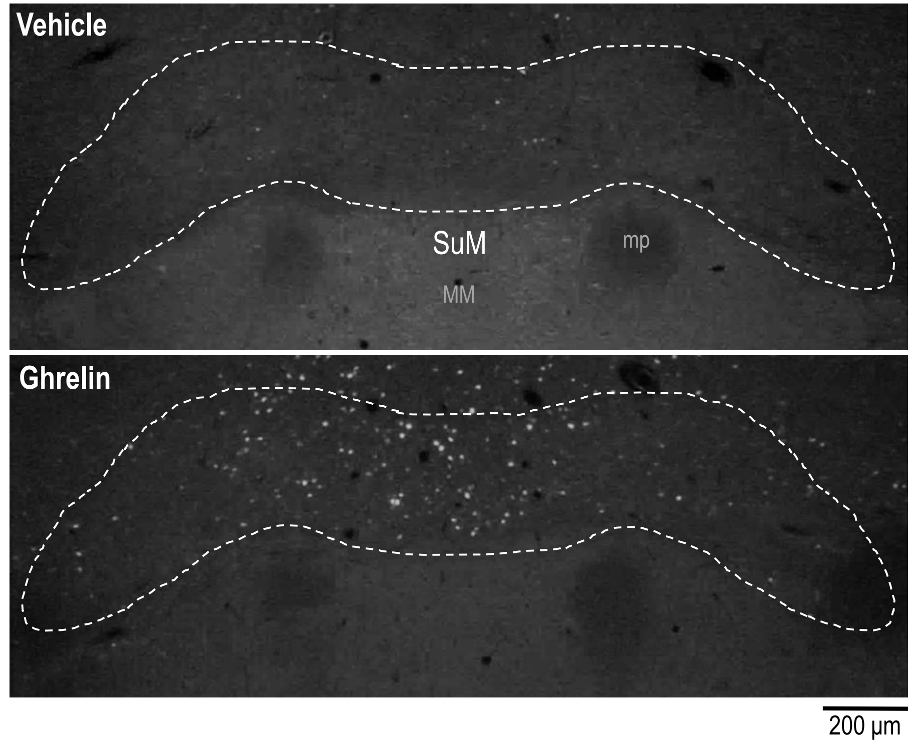
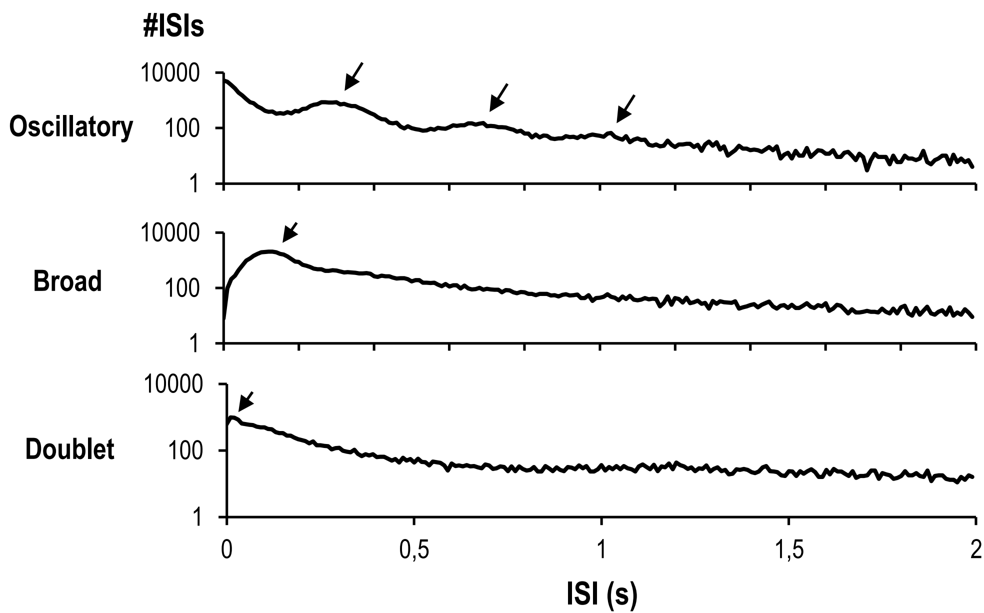
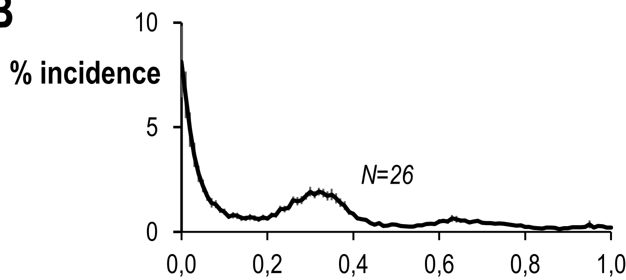
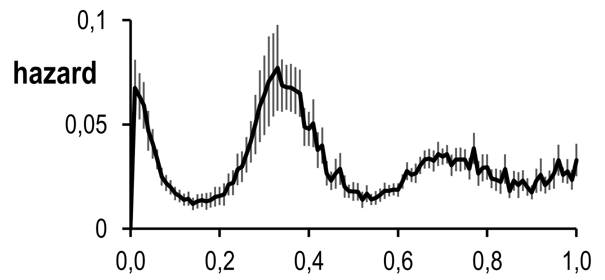
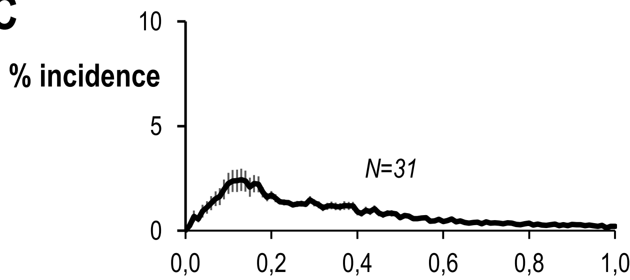


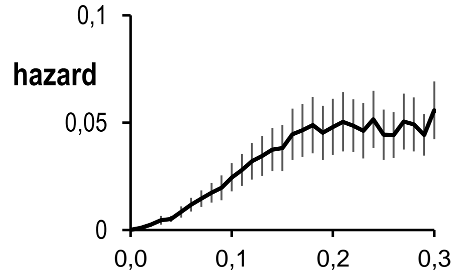
Figure 4

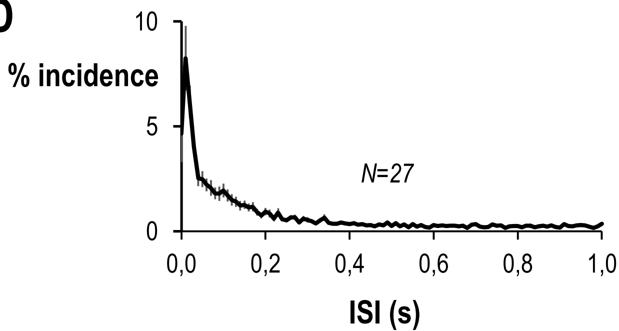












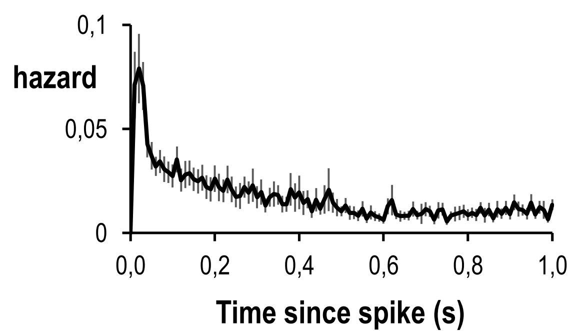


Figure 5

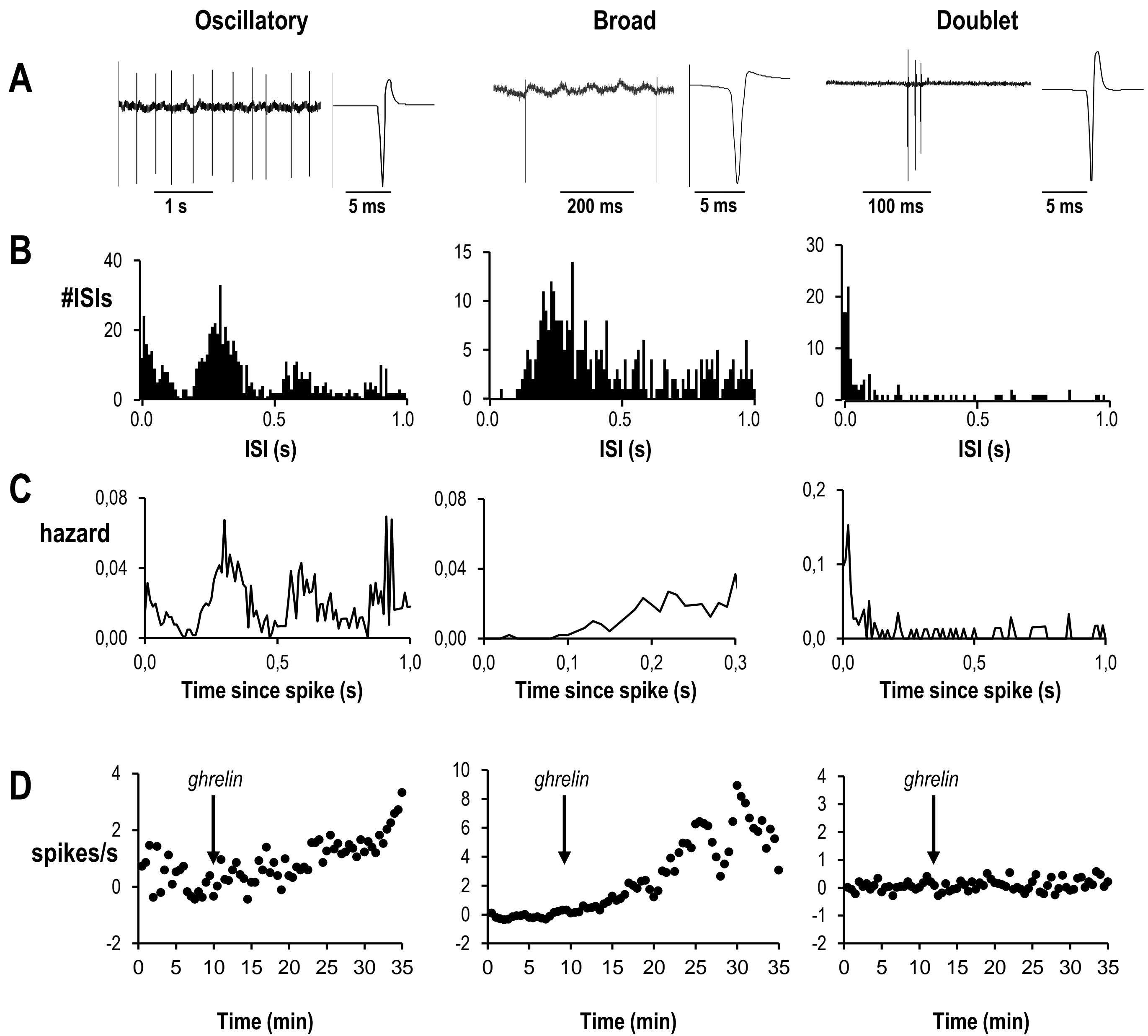
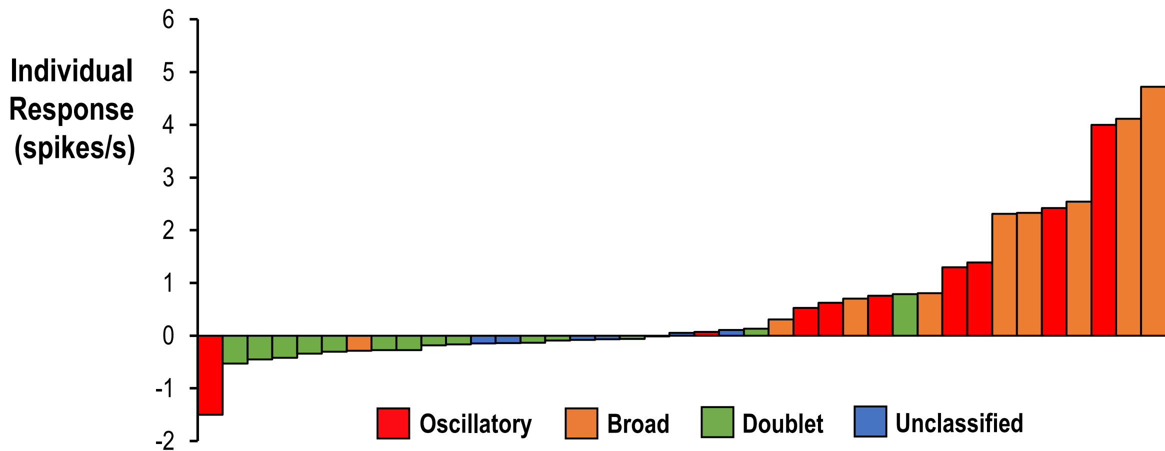
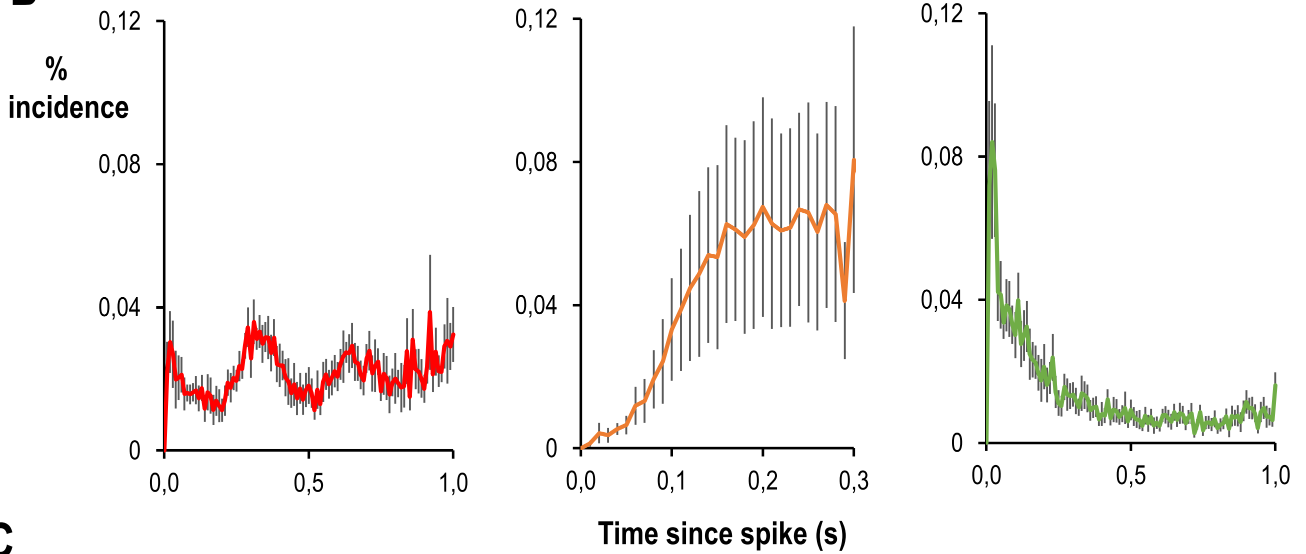


Figure 6





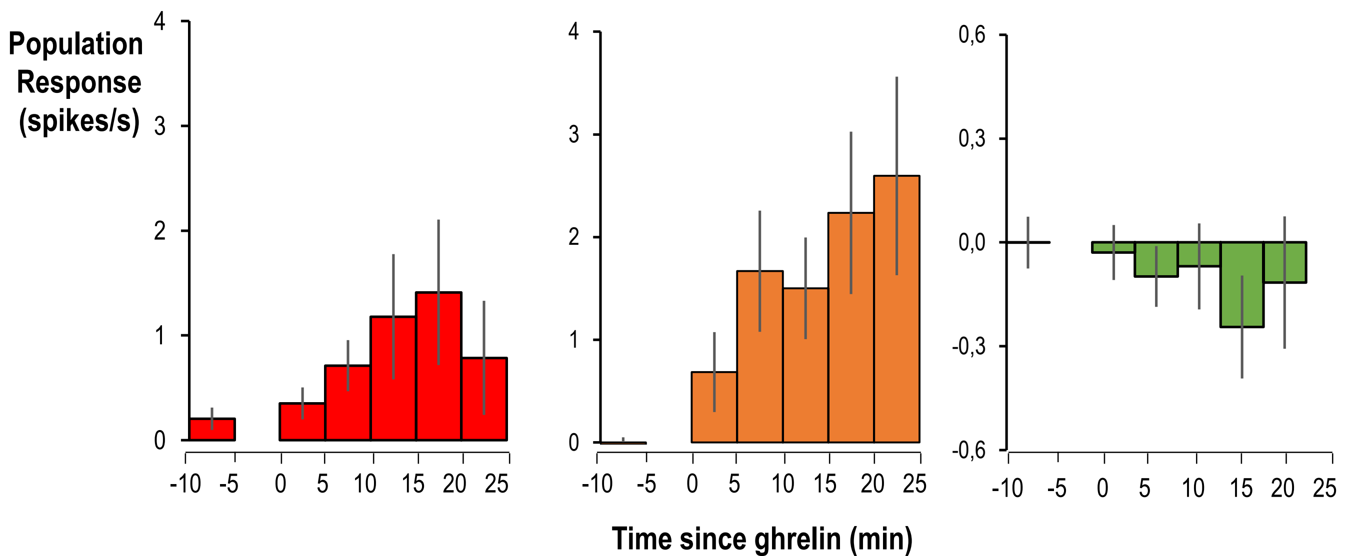


Figure 7