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 and 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 (eg, 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 anticipatory4 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 homeostasis, as well as those linked to reward, learning and memory.7 These include the hypothalamic ARC, PVN, LH, VMH and medial preoptic nucleus, as well as the VTA, amygdala, hippocampus, NAcc and various brain-stem areas, including the nucleus of the solitary tract, the laterodorsal tegmental, the 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, whereas 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 findings showing that ghrelin binding sites are present in the SuM17 and that an anorexigenic glucagon-like peptide 1-oestrone conjugate molecule can affect appetitive and consummatory behaviours at this site.11

The SuM has also been identified as a 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, the chemogenetic activation or inactivation of mouse SuM vglut2 neurones 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 and dopamine release follows a circadian pattern, with a peak at the onset of the active period of rats.22 Interestingly, these peaks are attenuated in rats made obese by a high-fat diet, indicating a potential sensitivity of 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 investigated the influence of food as a naturally rewarding and re-inforcing stimulus at this site. Using rats, we explored whether the neuronal activity of SuM cells (assessed by Fos mapping) is increased by peripheral 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 food reward.4,23 In addition, we aimed to determine whether i.v. ghrelin administration would modulate 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 site17 led us to explore the possibility that ghrelin has direct effects, as assessed in the present study by measuring the effect of intra-SuM ghrelin delivery on the feeding response.

## 2 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 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 djurförsöksnämnd (ethical permit 45-2014).

All studies were performed on adult male Sprague-Dawley rats aged 8-10 weeks housed in a 12:12 hour light/dark cycle (lights on 07:00 AM) at 20 ± 1°C, with water and standard diet (RM1; Special Diet Services, Witham, UK; or Teklad Global 16% Protein Rodent Diet; Envigo, Madison, WI, USA) available ad lib unless otherwise stated.

### 2.1 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 7 days. During this time, standard diet was available for 3 hours during the light phase (either 1:00 PM to 4:00 PM or 2:00 PM to 5:00 PM). It is well-established that rats can learn to anticipate periods of food access 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 hours 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 minutes after the beginning of the expected scheduled food access period (2:30 PM or 3:30 PM) and the SF-Unexp-Refed group perfused-fixed 90 minutes after the beginning of the unexpected food access period (10:30 AM to 11:30 AM). Rats were administered with sodium pentobarbitone (200 mg/kg) i.p. and transcardially perfused with ice-cold 0.9% hepaminised saline and 4% w/v paraformaldehyde in 0.1 mol L⁻¹ 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, cryo-protected in PB containing 30% w/v sucrose, frozen on dry ice and cut serially on a freezing microtome at 4 μm in the coronal plane. Free-floating SuM-containing sections were procured for c-Fos-like immunoreactivity using the 3,3′-diaminobenzidine (DAB)-hydrogen peroxide method, where they were incubated with an anti-c-Fos rabbit primary antibody (#226 003; Synaptic Systems, Göttingen, Germany; dilution 1:100 000), biotinylated horse anti-rabbit immunoglobulin (Ig)G secondary antibody (BA-1100; Vector Laboratories, Burlingame, CA, USA; dilution 1:500), avidin-biotin complex (ABC) (PK-6100; VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA; dilution 1:500), avidin-biotin complex (ABC) (PK-6100; VECTASTAIN Elite ABC Kit, Vector Laboratories) and a DAB, nickel and hydrogen peroxide solution, as described previously.²³ Brain sections were mounted on gelatin-coated slides, dehydrated in increasing concentrations of alcohol (70%-100%) followed by xylene (100%), coverslips applied with DPX mounting medium and imaged using a DMR reflected light microscope (Leica Microsystems, Wetzlar, Germany). 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 four 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 were calculated.

2.2 | Experiment 2: c-Fos expression in the SuM in ad lib.-fed rats scheduled-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 minutes each day for 7 days. SCM (Nestlé, Gatwick, 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 hours 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 (01:00 PM; “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 hours earlier than in the 7-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 hour following the end of the expected SCM access period (02:15 PM) and the Unexp-SCM groups were perfused 1 hour following the end of the unexpected SCM access period (10:15 AM). 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.

2.3 | 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 that received an i.p. injection of saline (n = 5) and a “Ghrelin” group that received an i.p. injection of ghrelin (n = 7; 110 μg kg⁻¹; #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 anaesthetised with Rompun® vet (10 mg kg⁻¹; Bayer, Leverkusen, Germany) and Ketaminol® vet (75 mg kg⁻¹; Intervet, Boxmeer, Netherlands), perfused transcardially with heparinised 0.9% saline followed by 4% paraformaldehyde in 0.1 mol L⁻¹ PB. The brains were post-fixed overnight at 4°C in 4% paraformaldehyde solution containing 15% sucrose and cryoprotected in 0.1 mol L⁻¹ 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 (dilution 1:20 000; Ab-5 (4-17) Rabbit pAb, PC38; Calbiochem, San Diego, CA, USA) for 2 days at 4°C, followed by Alexa Fluor 488 goat anti-rabbit secondary antibody (dilution 1:250; IgG[H+L]; A-11008; Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature before being mounted onto glass slides and coverslipped with ProLong® Diamond Antifade mountant (Life Technologies, Carlsbad, CA, USA). Images of the SuM-containing sections were acquired using a DM1B fluorescence microscope (Leica Microsystems). 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 four brain sections between bregma −4.36 to −4.68 mm under blinded conditions.

2.4 | 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 approximately 350 g; 84 cells) under urethane anaesthesia (ethyl carbamate, 1.3 g kg⁻¹ i.p.), using...
conventional extracellular recording techniques. A cannula was inserted into the femoral vein for i.v. injections. The rats were tra cheotomised and then 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 of approximately 1 μm) filled with 1.5% neurobiotin in 0.25 mol L⁻¹ 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 minutes under 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 minutes before ghrelin injection was compared with the rate over a 20-minute period starting 5 minutes after and ending 25 minutes after injection. We set a change of +0.5 spikes s⁻¹ as an arbitrary threshold above which the cell was considered as activated by ghrelin, and a change of −0.3 spikes s⁻¹ as the 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-seconds 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 minutes period starting 5 minutes after and ending 25 minutes after ghrelin injection was tested statistically to determine whether, as a subpopulation response, this change is statistically different from 0. For this, we used a Wilcoxon signed-rank test. P < 0.05 was considered statistically significant.

For all cells, interspike interval (ISI) histograms were constructed in 10 milliseconds bins from at least 10 minutes of stationary spontaneous discharge activity using spike2 (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 milliseconds bins using 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 milliseconds 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 for a sufficient length of time to be able to determine whether ghrelin induced a significant response or not, we applied anodal current pulses (200 milliseconds, 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 minutes. One neurone was labelled in each rat. At the end of the electrophysiology experiments, the rats were killed with an overdose of sodium pentobarbitone and perfused. Brains were post-fixed, cryoprotected and sectioned coronally at 44 μm, as described for Experiment 1. Floating sections were incubated with streptavidin conjugated to Alexa Fluor 594 and a DMRB fluorescence microscope (Leica Microsystems) was used to visualise the presence of neurobiotin.

2.5 | Experiment 5: Food intake after intra-SuM ghrelin administration

Male rats (250-310 g) were deeply anaesthetised by i.p. injection of a combination of Rompun® vet (10 mg kg⁻¹; Bayer) and Ketaminol® vet (75 mg kg⁻¹; 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 coordinates used were: anteroposterior: −4.8 mm; mediolateral: ±0.7 mm; dorsoventral: −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 s.c. with an analgesic (Rimadyl®, Orion Pharma Animal Health, Sollentuna, Sweden), housed singly 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 artificial cerebrospinal fluid; Tocris) on different days, separated by a wash-out day, in a cross-over design, so that 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, 6 and 24 hours post-intra-SuM injection. At the end of the study, the animals were deeply anaesthetised with isoflu rane (Baxter, Deerfield, IL, 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.

2.6 | Statistical analysis

Where appropriate, data are expressed as the mean ± SEM. Statistical analysis was conducted using spss, version 25 (IBM Corp.,
Armonk, NY, USA) or Prism, version 6 (GraphPad Software Inc., San Diego, CA, USA). 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 performed.
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. \( P < 0.05 \) was considered statistically significant.

3 | RESULTS

3.1 | 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 per section; SF-Unfed: 364.3 ± 20.7 c-Fos+ nuclei per 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 per section; SF-Unexp-Refed: 383.9 ± 54.5 c-Fos+ nuclei per section; both \( P > 0.99 \)) (Figure 1).

3.2 | 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 per section; Exp-SCM: 199.1 ± 18.9 c-Fos+ nuclei per section; \( P = 0.048 \)) (Figure 2). Compared to controls, rats consuming SCM at an unexpected time also had a significantly higher number of SuM c-Fos+ nuclei (Unexp-SCM: 299.7 ± 23.0 c-Fos+ nuclei per 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-Received: 14.3 ± 3.0 c-Fos+ nuclei per section, \( n = 7 \); Expected-Received: 20.3 ± 4.0 c-Fos+ nuclei per section, \( n = 8 \); \( P = 0.23 \)).

3.3 | 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 per section; Ghrelin: 61.2 ± 14.9 c-Fos+ nuclei per section; \( P = 0.018 \)) (Figure 3).

3.4 | 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\(^{-1}\)) recorded at depths consistent with localisation in the SuM. From their ISI distributions and hazard functions, we classified these cells into three groups (Figures 4 and 5), as outlined below.

One group of 26 cells, that we call “oscillatory cells,” fired at 3.1 ± 0.54 spikes s\(^{-1}\). These had multimodal ISI distributions, with one narrowly defined mode at <20 milliseconds, a second broader mode peaking at 270 milliseconds in the summed distribution and a third mode peaking at 660 milliseconds (the third mode was only apparent in cells that had a sufficiently large number of ISIs exceeding 400 milliseconds) (Figure 4A). These modes are apparent in the sum of the ISI distributions, which also shows a mode at 1040 milliseconds (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\(^{-1}\). These had unimodal ISI distributions, with one broad mode peaking at approximately 140 milliseconds in the sum of the ISI distributions (Figure 4A,C). 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 after hyperpolarisation.\(^{27}\)
A third group of 31 cells, which we call "doublet" cells (again following the terminology used for VMH cells\textsuperscript{30}) fired at 2.3 ± 0.42 spikes s\(^{-1}\). These had unimodal distributions with a single mode at <30 ms, and subsequent modes (arrowed) at approximately 350 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.

Nine "oscillatory" cells were tested, firing at an initial rate of 1.6 ± 0.5 spikes s\(^{-1}\). The firing rate of seven of these cells increased by >0.5 spikes s\(^{-1}\) and that of one cell fell by 1.5 spikes s\(^{-1}\). Overall, the mean firing rate of the group increased by 1.1 ± 0.5 spikes s\(^{-1}\) (Figures 5D and 6C), although 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\(^{-1}\). The firing rate of seven of these cells increased by >0.5 spikes s\(^{-1}\), and the mean firing rate of this group increased significantly (\(P = 0.009\)) by 2.0 ± 0.6 spikes s\(^{-1}\) (Figures 5D and 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\(^{-1}\), and the mean firing rate fell significantly (\(P = 0.025\)) after ghrelin by 0.17 ± 0.09 spikes s\(^{-1}\). Another seven cells were tested that were firing too slowly (at 0.3 ± 0.1 spikes s\(^{-1}\)) 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\(^{-1}\) (Figures 5D and 6C).
Experiment 5: Food intake with intra-SuM ghrelin

Compared to vehicle, intra-SuM injection of ghrelin increased food intake at 3 hours 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 hours 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 hours post-intra-SuM ghrelin injection for either dose (data not shown).

4 | DISCUSSION

The present study identifies the SuM as a brain area that is activated when rats are hungry and/or anticipating food, as well as being activated 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
FIGURE 6  Responses of recorded supramammillary nucleus (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 seven slow (unclassified) cells in blue. Each bar represents one SuM neurone, and its response averaged over the 20-minute period between 5 and 25 minutes after ghrelin injection. B, Mean ± SEM hazard functions of the nine “oscillatory” cells (left), the nine “broad” cells (middle) and the 14 “doublet” cells (right). C, Mean ± SEM responses of these cells to i.v. ghrelin, calculated as the mean difference in firing rate (in 5-minute bins) from the firing rate in the 5 minutes before injection.
demonstrate that central ghrelin injection directed at the SuM drives a feeding response.

We carried out scheduled refeeding 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. Refeeding 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, although 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 and motivated behaviour 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

**FIGURE 7** Effect of injection of ghrelin (0.5 and 1 µg doses) directed at the supramamillary nucleus (SuM) on chow intake (g). Data are shown as the mean ± SEM (n = 13). A, Representative brain section showing SuM injection site (ink injection; right) and corresponding rat brain atlas section (left). Bregma −4.68 mm.24 B, Cumulative chow intake at 3 hours 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 hours post intra-SuM ghrelin injection: repeated measures one-way ANOVA ($F_{1.8,21.8} = 15.96, P < 0.0001$) with Bonferroni (*$P < 0.05$, ****$P < 0.0001$)
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 the 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. The SuM is bordered by multiple nuclei, including the LH, perifornical nucleus, posterior hypothalamic area and VTA. Differences in cellular organisation between the SuM and bordering regions demonstrate that the SuM is a defined nucleus. Immunohistochemical studies have shown SuM neurones to express tyrosine hydroxylase, cholecystokinin, substance P and vasoactive intestinal peptide, alongside other peptides. 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 shown previously that gavage of SCM activates magnocellular oxytocin cells. Oxytocin can be released into the brain from central dendrites to act at distant sites, oxytocin-immunoreactive nerve fibres are reported to enter the SuM and oxytocin binding sites are present in the rat SuM. In acute brain slice preparations, oxytocin receptor agonists activate approximately half of SuM cells tested. 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 the well-established roles of ghrelin 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. In the present study, we report 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 because they show a rhythmic firing pattern, consistent with an underlying sinusoidal oscillation in excitability at a frequency of approximately 4 Hz (ie 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. SuM neurones project to the dentate gyrus and CA2 area of the hippocampus, and pharmacological blockade of the SuM in conscious or anaesthetised rats disrupts hippocampal theta rhythm. 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. An in vivo electrophysiological study in the SuM and mamillary body of the urethane-anaesthetised rat studied the relationship between the electrical activity of single SuM cells and the hippocampal electroencephalogram. It was found 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 controversial. 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. 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. 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 and, 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, as well as investigate the effects of appetite-related hormones on the functional connectivity of these circuits.

The electrophysiological approach used in the present study requires that the recorded cells have some spontaneous activity at baseline, making it possible to observe both inhibitory and excitatory responses, although, inevitably, this 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, there is heterogeneity in the response of SuM cells. However, responses in the SuM were significant, long lasting and comparable in magnitude to the responses of ARC neurones to ghrelin receptor agonists in vivo, indicating that the SuM is a relevant ghrelin-responsive area of the hypothalamus.

Peripheral ghrelin injection is demonstrated to alter the electrical activity of SuM cells recorded in anaesthetised rats and to increase the number of SuM cells expressing c-Fos. This is compelling
evidence for the SuM comprising a relevant brain structure with respect to the neurobiological effects of ghrelin because it is also activated at times when endogenous ghrelin levels are high. Circulating ghrelin is known to access the brain. For example, previous studies have shown that peripheral ghrelin binds to the ARC (via the median eminence) and the hippocampus. Because centrally injected ghrelin binds to the SuM, the possibility exists that peripheral ghrelin could access the SuM and activate these cells directly.

Although it is difficult to map the neural circuitry engaged by peripheral ghrelin that causes 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 as via heterodimerisation with other receptors or indeed by the recently discovered endogenous antagonist, LEAP2. The fact that GHSR1A has constitutive activity means that ghrelin-responsive pathways may not require ghrelin for their activation. To explore the 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 the orexigenic effect of ghrelin. It would be important to determine how other ghrelin-responsive targets connect to the SuM for a deeper understanding of the neuronal network mediating the behavioural effects of ghrelin. 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, comprising 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 of whether food was presented or absent at the expected time. This was previously observed in other hypothalamic nucleus important in feeding behaviours (ARC, LH, VMH and dorsomedial hypothalamus). Moreover, given that circulating levels of ghrelin are correlated with food anticipation and that central ghrelin injection increases behaviours associated with food anticipation, the SuM might be activated, at least in part, by ghrelin during food anticipation. This is in contrast, for example, with the AgRP neurones in the ARC that are activated when mice anticipate food, whereas rapidly suppressed when food becomes available.

In summary, the data obtained in the present study 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, as ghrelin.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

AUTHOR 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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REFERENCES

1. Morton GJ, Meek TH, Schwartz MW. Neurobiology of food intake in health and disease. Nat Rev Neurosci. 2014;15:367-378.
2. Kanoski SE, Grill HJ. Hippocampus contributions to food intake control: mnemonic, neuroanatomical, and endocrine mechanisms. Biol Psychiatry. 2017;81:748-756.
3. Skibicka KP, Hansson C, Egecioglu E, Dickson SL. Role of ghrelin in food reward: impact of ghrelin on sucrose self-administration and mesolimbic dopamine and acetylcholine receptor gene expression. Addict Biol. 2012;17:95-107.
4. Verhagen LA, Egecioglu E, Luijendijk MC, Hillebrand JJ, Adan RA, Dickson SL. Acute and chronic suppression of the central ghrelin signaling system reveals a role in food anticipatory activity. Eur Neuropsychopharmacol. 2011;21:384-392.
5. Schele E, Bake T, Rabasa C, Dickson SL. Centrally administered ghrelin acutely influences food choice in rodents. PLoS ONE. 2016;11:e0149456.
6. Wren AM, Small CJ, Abbott CR, et al. Ghrelin causes hyperphagia and obesity in rats. Diabetes. 2001;50:2540-2547.
7. Zigman JM, Jones JE, Lee CE, Saper CB, Elmquist JK. Expression of ghrelin receptor mRNA in the rat and the mouse brain. J Comp Neurol. 2006;494:528-548.
8. Alvarez-Crespo M, Skibicka KP, Faraks I, et al. The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence. PloS ONE. 2012;7:e46321.
9. Kanoski SE, Fortin SM, Ricks KM, Grill HJ. Ghrelin signaling in the ventral hippocampus stimulates learned and motivational aspects of feeding via PI3K-Akt signaling. Biol Psychiatry. 2013;73:915-923.
10. Skibicka KP, Hansson C, Alvarez-Crespo M, Friberg PA, Dickson SL. Ghrelin directly targets the ventral tegmental area to increase food motivation. Neuroscience. 2011;180:129-137.
11. Vogel H, Wolf S, Rabasa C, et al. GLP-1 and estrogen conjugate acts on the supramammillary nucleus to reduce food-reward and body weight. Neuropharmacology. 2016;110:396-406.
12. Pan WX, McNaughton N. The supramammillary area: its organization, functions and relationship to the hippocampus. Prog Neurobiol. 2004;74:127-166.
13. Ikemoto S. The supramammillary nucleus mediates primary reinforcement via GABA(A) receptors. Neuropsychopharmacology. 2005;30:1088-1095.
14. Shin R, Ikemoto S. Administration of the GABA receptor antagonist picrotoxin into rat supramammillary nucleus induces c-Fos in reward-related brain structures. Supramammillary picrotoxin and c-Fos expression. BMC Neurosci. 2010;11:101.
15. Ikemoto S, Witkin BM, Zangen A, Wise RA. Rewarding effects of AMPA administration into the supramammillary or posterior hypothalamic nuclei but not the ventral tegmental area. J Neurosci. 2004;24:5758-5765.
16. Kolodziej J, Lippert M, Angenstein F, et al. SPECT-imaging of activity-dependent changes in regional cerebral blood flow induced by electrical and optogenetic self-stimulation in mice. NeuroImage. 2014;103:171-180.
17. Cabral A, Fernandez G, Perello M. Analysis of brain nuclei accessible to ghrelin present in the cerebrospinal fluid. Neuroscience. 2013;253:406-415.
18. Stagkourakis S, Spigolon G, Williams P, Protzmann J, Fisone G, Broberger C. A neural network for intermale aggression to establish social hierarchy. Nat Neurosci. 2018;21:834-842.
19. Pedersen NP, Ferrari L, Venner A, et al. Supramammillary glutamate neurons are a key node of the arousal system. Nat Commun. 2017;8:1405.
20. Renouard L, Billwiller F, Ogawa K, et al. The supramammillary nucleus and the caudustinate activate the cortex during REM sleep. Sci Adv. 2015;1:e1400177.
21. Ito M, Shirao T, Doya K, Sekino Y. Three-dimensional distribution of Fos-positive neurons in the supramammillary nucleus of the rat exposed to novel environment. Neurosci Res. 2009;64:397-402.
22. Luo S, Zhang Y, Ezroki M, Li Y, Tsaï TH, Cincotta AH. Circadian peak dopaminergic activity response at the biological clock pacemaker (suprachiasmatic nucleus) area mediates the metabolic responsiveness to a high-fat diet. J Neuroendocrinol. 2018;30:e12563.
23. Merkstein M, Brans MA, Luijendijk MC, et al. Ghrelin mediates anticipation to a palatable meal in rats. Obesity (Silver Spring). 2012;20:963-971.
24. Patton DF, Mistlberger RE. Circadian adaptations to meal timing: neuroendocrine mechanisms. Front Neurosci. 2013;7:185.
25. Hume C, Sabatier N, Menzies J. High-Sugar, but Not High-Fat, Food Activates Supraoptic Nucleus Neurons in the Male Rat. Endocrinology. 2017;158:2200-2211.
26. Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. Waltham, MA: Academic Press; 2006.
27. Sabatier N, Leng G. Spontaneous discharge characteristic of neurons in the ventromedial nucleus of the rat hypothalamus in vivo. Eur J Neurosci. 2008;28:693-706.
28. Sabatier N, Brown CH, Ludwig M, Leng G. Phasic spike patterning in rat supraoptic neurons in vivo and in vitro. J Physiol. 2004;558:161-180.
29. Pinault D. A novel single-cell staining procedure performed in vivo under electrophysiological control: morpho-functional features of juxtacellulary labeled thalamic cells and other central neurons with bicynotin or Neurobiotin. J Neurosci Methods. 1996;65:113-136.
30. Skibicka KP, Shirazi RH, Rabasa-Papi C, et al. Divergent circuitry underlying food reward and intake effects of ghrelin: dopaminergic VTA-accumbens projection mediates ghrelin’s effect on food reward but not food intake. Neuropharmacology. 2013;73:274-283.
31. Geeraets M, Nieuwenhuys R, Veening JG. Medial forebrain bundle of the rat: IV. Cytoarchitecture of the caudal (lateral hypothalamic) part of the medial forebrain bundle bed nucleus. J Comp Neurol. 1990;294:537-568.
32. Shepard PD, Mihailoff GA, German DC. Anatomical and electrophysiological characterization of presumed dopamine-containing neurons within the supramammillary region of the rat. Brain Res Bull. 1988;20:307-314.
33. Serogy K, Tsuruo Y, Hofkelt T, et al. Further analysis of presence of peptides in dopamine neurons. Cholecystokinin, peptide histidine-isoleucine/vasoactive intestinal polypeptide and substance P in rat supramammillary region and mesencephalon. Exp Brain Res. 1988;72:523-534.
34. Ludwig M, Apps D, Menzies J, Patel JC, Rice ME. Dendritic Release of Neurotransmitters. Compr Physiol. 2016;7:235-252.
35. Cumbers MR, Chung ST, Wakerley JB. A neuromodulatory role for oxytocin within the supramammillary nucleus. Neuroptides. 2007;41:217-226.
36. Kremarik P, Freund-Mercier MJ, Stoeckel ME. Oxytocin and vasopressin binding sites in the hypothalamus of the rat: histoautoradiographic detection. Brain Res Bull. 1995;36:195-203.
37. Korotkova T, Ponomarenko A, Monaghan CK, et al. Reconciling the different faces of hippocampal theta: the role of theta oscillations in cognitive, emotional and innate behaviors. Neurosci Biobehav Rev. 2018;85:65-80.
38. Vertes RP. Major diencephalic inputs to the hypothalamus: supramammillary nucleus and nucleus reuniens. Circuity and function. Prog Brain Res. 2015;219:121-144.
39. Aranda L, Begega A, Sanchez-Lopez J, Aguirre JA, Arias JL, Santin LJ. Temporary inactivation of the supramammillary area impairs spatial working memory and spatial reference memory retrieval. Physiol Behav. 2008;94:322-330.
40. Kocsis B, Vertes RP. Phase relations of rhythmic neuronal firing in the supramammillary nucleus and mammillary body to the hippocampal theta activity in urethane anesthetized rats. Hippocampus. 1997;7:204-214.
41. Kocsis B, Vertes RP. Characterization of neurons of the supramammillary nucleus and mammillary body that discharge rhythmically with the hippocampal theta rhythm in the rat. J Neurosci. 1994;14:7040-7052.
42. Jones MW, Wilson MA. Theta rhythm coordinate hippocampal-frontal interactions in a spatial memory task. PloS Biol. 2005;3:e402.
43. Dickson SL, Leng G, Robinson IC. Systemic administration of growth hormone-releasing peptide activates hypothalamic arcuate neurons. Neuroscience. 1993;53:303-306.
44. Schaeffer M, Langlet F, Lafort C, et al. Rapid sensing of circulating ghrelin by hypothalamic appetite-modifying neurons. Proc Natl Acad Sci USA. 2013;110:1512-1517.
45. Diano S, Farr SA, Benoit SC, et al. Ghrelin controls hippocampal spine synapse density and memory performance. Nat Neurosci. 2006;9:381-388.
46. Schellekens H, Dinan TG, Cryan JF. Taking two to tango: a role for ghrelin receptor heterodimerization in stress and reward. Front Neurosci. 2013;7:148.
47. Ge X, Yang H, Bednarek MA, et al. LEAP2 is an endogenous antagonist of the ghrelin receptor. *Cell Metab.* 2018;27:461-469.e6.

48. Holst B, Cygankiewicz A, Jensen TH, Ankersen M, Schwartz TW. High constitutive signaling of the ghrelin receptor—identification of a potent inverse agonist. *Mol Endocrinol.* 2003;17:2201-2210.

49. Johnstone LE, Fong TM, Leng G. Neuronal activation in the hypothalamus and brainstem during feeding in rats. *Cell Metab.* 2006;4:313-321.

50. Betley JN, Xu S, Cao ZFH, et al. Neurons for hunger and thirst transmit a negative-valence teaching signal. *Nature.* 2015;521:180-185.

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