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Ghrelin and orexin interact to increase meal size through a descending hippocampus to hindbrain signaling pathway

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Abstract

Background—Memory and cognitive processes influence the amount of food consumed during a meal, yet the neurobiological mechanisms mediating these effects are poorly understood. The hippocampus (HPC) has recently emerged as a brain region that integrates feeding-relevant biological signals with learning and memory processes to regulate feeding. Here we investigate whether the gut-derived hormone, ghrelin, acts in the ventral HPC (vHPC) to increase meal size through interactions with gut-derived satiation signaling.

Methods—Interactions between vHPC ghrelin signaling, gut-derived satiation signaling, feeding, and interoceptive discrimination learning were assessed via rodent behavioral neuropharmacological approaches. Downstream neural pathways were identified using transsynaptic virus-based tracing strategies.

Results—Results reveal that vHPC ghrelin signaling counteracts the food intake-reducing effects produced by various peripheral biological satiation signals, including cholecystokinin, exendin-4 (a glucagon-like peptide-1 receptor agonist), amylin, and mechanical distension of the stomach. Results further show that vHPC ghrelin signaling may attenuate satiation processing by producing interoceptive cues that generalize to a perceived state of energy deficit. Neuroanatomical tracing results identify a multi-order connection from vHPC neurons to lateral hypothalamic area (LHA) orexin (aka, hypocretin)-producing neurons that project to the laterodorsal tegmental nucleus (LDTg) in the hindbrain. Lastly, results show that vHPC ghrelin signaling increases spontaneous meal size via downstream orexin receptor signaling in the LDTg.

Conclusions—We conclude that vHPC ghrelin signaling increases meal size by counteracting the efficacy of various gut-derived satiation signals. These effects occur via downstream orexin

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signaling to the hindbrain LDTg, thereby highlighting a novel hippocampus-hypothalamus-hindbrain pathway regulating meal size control.

Keywords

obesity; brainstem; lateral hypothalamus; food intake; reward; satiation

Introduction

The predominance of research on the neural control of energy balance has focused on the hypothalamus as a primary center regulating food intake and energy expenditure (1, 2). However, emerging findings highlight the importance of hindbrain and other extra-hypothalamic regions in food intake regulation, particularly in the control of cognitive aspects of feeding behavior related to reward and memory processes (3–7). The hippocampus (HPC), a brain region traditionally associated with declarative and visuospatial memory function (8, 9), has recently emerged as a critical brain region in the control of food intake and feeding-related cognitive processes [see (10–12) for review]. Moreover, evidence suggests that the HPC also contributes to satiation/meal size control, as HPC neurons are activated by within-meal gastrointestinal (GI) satiation signals, including cholecystokinin, gastric distension, intra-gastric nutrient infusion, and electrical stimulation of the gastric branch of the vagus nerve (13–16). The neurobiological mechanisms through which satiation signals communicate with and are influenced by HPC neural processing are unknown. The interaction between satiation signaling and the HPC may involve endocrine pathways, as leptin, GLP-1, and ghrelin influence feeding behavior, in part, via action in HPC neurons (17–19).

Ghrelin is a stomach-derived 28-amino acid “hunger hormone” that increases appetite and food intake via its seven transmembrane G-protein-coupled receptor, the type 1a growth hormone secretagogue receptor (GHSR1a) (20, 21). Ghrelin levels are elevated during energy restriction (22–24) and decrease following a meal (25–26). Previous work revealed that CNS ghrelin signaling promotes food intake via interactions with GI-derived vagally-mediated satiation signals. For example, peripheral ghrelin administration increases gastric emptying rate in both humans and rodent models (27, 28), and the hindbrain area postrema is one site mediating these effects (29). Further, the food restriction-induced elevation in circulating ghrelin levels is abolished by subdiaphragmatic vagotomy (30). Given that meal size is considered to be a primary determinant of overall caloric intake (31, 32), it is important to understand the neurobiological mechanisms through which ghrelin regulates satiation signaling.

GHSR1a is expressed in the dentate gyrus, CA1, CA2, and CA3 regions of the hippocampal formation, (19, 33–36) and GHSR1a activation in the ventral hippocampus subregion (vHPC) increases food intake (37, 38). However, the mechanisms mediating these outcomes are poorly understood. Based on research discussed above identifying a role for HPC ghrelin signaling in food intake control, combined with several recent reports that HPC neurons respond to vagally-mediated gastrointestinal signals (13, 14, 16, 39), we hypothesize that HPC ghrelin signaling promotes food intake, in part, by reducing the capacity of GI-derived

satiation signals to terminate a meal. Moreover, in light of previous reports that CNS ghrelin signaling engages neurons in the lateral hypothalamic area (LHA) that produce the neuropeptide, orexin (aka, hypocretin), and that CNS ghrelin and orexin interact to increase feeding (19, 40, 41), we further hypothesize that HPC ghrelin counteracts satiation processing via downstream LHA orexin signaling.

Methods and Materials

Animals

Adult male Sprague-Dawley rats (Envigo; 320–450g on arrival) were individually housed with ad libitum access (except where noted) to water and chow (LabDiet 5001, LabDiet, St. Louis, MO) on 12 hr:12 hr light/dark cycle (lights on at 06:00h). All procedures involving animals were approved by the University of Southern California Institute of Animal Care and Use Committee.

Experiment 1: Interactions between vHPC ghrelin and gut-derived within-meal satiation signals

General Design—Following 24-hr food restriction, rats with bilaterally implanted vHPC cannulae received injections of either aCSF/vehicle or a subthreshold dose of ghrelin when injected alone (30 pmol/ 200 nl; dose selected from pilot work) 45 mins before intraperitoneal administration of a pharmacological treatments [vs. i.p. 0.9% sterile saline (1 ug/kg) control] or 1 hr before methylcellulose gavage (see Supplementary Methods for cannula implantation and drug preparation parameters). Food was returned immediately after i.p. injections/gavage. Treatments were separated by 2–3 intervening days using a counterbalanced within-subjects design (Experiments 1a, 1b) or using a counterbalanced mixed design with vHPC drug as a between-subjects variable and i.p. drug as a within-subjects variable (Experiments 1c, 1d). Chow intake and spillage were recorded at 30 mins, 1, 2, and 4 hours after injections.

Experiment 1a: CCK (n=8)—This food intake-reducing dose of i.p. CCK (3ug/kg, ip) was previously shown to depend on intact vagal afferent signaling (42).

Experiment 1b: methylcellulose gavage for mechanical stomach distension (43, 44)—2% methylcellulose (MC; viscosity 400 cP, Sigma-Aldrich) in 0.2% Tween-80 (Sigma-Aldrich) dissolved in warm DI water was stirred at room temperature. Animals (n=11) received an oral gavage administration of 15 mL of 2% MC or a sham gavage (no infusions).

Experiment 1c: Exendin-4 (n=20)—The Exendin-4 dose (3 ug/kg, ip) was selected because the food intake-reducing effects of i.p. Exendin-4 at this dose are dependent on vagal afferent signaling (45).

Experiment 1d: Amylin (n=23)—The amylin dose (50 ug/kg, ip) was selected because this dose reduces food intake via non-vagal mechanisms (46).

Experiment 2: Deprivation intensity discrimination (DID)

Experiment 2a: Deprivation intensity discrimination (DID) training—The DID task involves rats learning to use interoceptive energy status cues (0 vs. 24 h food restriction) as discriminative stimuli for a sucrose reinforcement. Training procedures follow previous publications (16, 47–49) (see Supplementary Figure 2a). Rats with HPC lesions are impaired in learning this discrimination problem (49, 50), indicating that the HPC is critical for this type of interoceptive discrimination memory process.

Experiment 2b: DID testing—At the end of training, rats from both groups were tested on 2 days under 0-h food restriction state, with one 24-h food restriction day without treatment intervening between the two test days. The first test day took place one day after the last 24-h food restricted training day. Testing was conducted during extinction, such that feeder operated, but no sucrose pellets were delivered to either group. On each of two test days, rats with cannulae targeting the vHPC received bilateral administration of aCSF or ghrelin (150 pmol/200 nl) approximately 60 mins prior to being placed in conditioning chambers, using a within-subjects design with order of treatments counterbalanced across Groups 0+ and 24+.

Experiment 3: Identification of downstream targets of vCA1 to LHA projections

Experiment 3a: Neural tract tracing and immunohistochemistry—To drive expression of Cre recombinase in 1st order targets of vCA1 neurons, rats (n=5) received a unilateral 200nl pressure co-injection of AAV1-hSyn-Cre-WPRE-hGH and CTB-488 (CTB for postmortem injection site confirmation) in the vCA1 of the HPC. Next, animals received a unilateral 200nl pressure injection of a Cre-dependent anterograde tracer, AAV1-CAG-Flex-tdTomato-WPRE-rBG, in the lateral hypothalamic area (LHA) (see Supplemental Methods for coordinates). This Cre-mediated transsynaptic anterograde tracing approach allows us to identify downstream targets of vCA1 HPC to LHA second-order projections by mapping axons of LHA neurons that receive synaptic input from vCA1 neurons (16, 51). Following a 3-week survival period, animals housed in 6:00am lights off were both food restricted and received i.p. injections of saline (n=2) or CCK (n=3) 90-minutes before perfusion and tissue was harvest and processed as described in Supplementary Methods. Representative images for orexin protein expression in the LDTg obtained from these animals were confined to Swanson Atlas level 48–50 (52).

Experiment 3b: Fluorescence in situ hybridization (FISH) and immunohistochemistry—In control rats (n=3), tissue was harvested following transcardial perfusion for mRNA analyses (see Supplemental Methods for details). Representative images for GHSR1a and VGLUT1 mRNA expression in the vCA1 subregion of HPC and for ORX1-R and GLP1-R mRNA expression in the LDTg obtained from these animals were confined to Swanson Atlas levels 36–38 and 48–50 (52), respectively.

Experiment 4: Interactions between vHPC GHSR1a and LDTg ORX-1R signaling on spontaneous meal pattern parameters

Meal pattern analyses (measuring cumulative food intake, meal frequency, and meal size) were conducted in chambers equipped with automated food intake monitors (Med

Associates Inc.; see Supplementary Methods for details). Immediately prior to the dark cycle, animals received LDTg bilateral administration of either 50% DMSO/aCSF or a dose of an ORX-1R antagonist, SB-334867, subthreshold for food intake effects alone (1 ug/200 nl; dose based on (40) and our pilot work), immediately followed by vHPC bilateral administration of either aCSF or ghrelin (150 pmol/200 nl total, 75 pmol/100 nl per side; dose selected to have hyperphagic effects when administered alone (37)). Animals were then placed in the chamber for 15-hrs and food intake was continuously monitored. Treatments were counterbalanced using a within-subjects design and were separated by 2–3 days where animals were returned to their home cage between treatments.

Statistical Analyses

Statistical analyses used multifactorial repeated measures analysis of variance (rANOVA), except for Experiment 3 (one-way ANOVA) and Experiment 2a (unpaired Student t-tests). Fisher LSD (Experiment 2b) and Newman-Keuls (Experiments 1 and 4) post hoc comparisons were used when significant main effects and/or interactions were obtained. Results are presented as mean ± SE. Statistical significance was set at $p<0.05$. Statistical analyses were conducted with computer software (Statistica V7; Statsoft).

Results

Experiment 1: vHPC ghrelin signaling counteracts the food intake-reducing effects of meal-derived gastrointestinal satiation signals

In 24-hour food restricted animals, a subthreshold dose of ghrelin for feeding effects alone administered to the vHPC attenuates the food intake reduction during refeeding produced from the satiation signals CCK (i.p., 3ug/kg, Experiment 1a), gastric distension (Experiment 1b), Exendin-4 (3ug/kg, Experiment 1c), and amylin (50ug/kg. Experiment 1d) (Figure 1) (statistical results in Supplemental Results). Given that three of these peripheral meal-derived satiation signal treatments were designed based on vagus nerve requirement for food intake reduction (CCK, distension, Exendin-4) whereas one treatment's effects do not require the vagus (amylin), results indicate that the enhancement of meal size associated with vHPC ghrelin signaling involves interactions with both paracrine (vagal) and non-vagal endocrine pathways. Collective results from Experiment 1 demonstrate that vHPC ghrelin signaling, at a dose that has no effects on feeding alone, attenuates the anorexigenic effects of an array of vagally- and non-vagally-mediated within-meal satiation signals.

Experiment 2: vHPC ghrelin signaling produces interoceptive cues that generalize to 24-hr food restriction

Based on findings that an intact hippocampus is required for discriminating between low and high levels of food restriction in rats (49, 50), and that both peripheral (i.p.) and central administration of ghrelin produce an energy state that generalizes to 24-hr food restriction (48), we hypothesized that vHPC ghrelin signaling counteracts satiation signaling by producing interoceptive cues that generalize to a state of energy restriction. Indeed, results revealed that vHPC ghrelin administration in nonrestricted rats produced an interoceptive state similar to 24hr food restriction, as evident from increased anticipatory appetitive

responding in Group 24+ following ghrelin compared to aCSF, and vice versa for rats in Group 0+ (consistent with the performance for each group on 24-hr food-restricted training days at the end of the training phase) (statistical results in Supplemental Results). Overall, results show that vHPC ghrelin signaling counteracts satiation processing, in part, by producing an internal perception of energy deficit similar to that experienced following 24-hrs without food.

Experiment 3: Orexin neurons in the LHA are a relay connecting the vHPC (field CA1) to the LDTg

Based on our previous work showing that the orexigenic effects of unilateral vHPC ghrelin administration are abolished following contralateral lesion of the LHA (19), we hypothesized that the LHA is a monosynaptic downstream mediator of vHPC ghrelin effects on meal size control. We utilized a transsynaptic anterograde tracing approach combined with immunohistochemistry and in situ hybridization to confirm that vCA1 pyramidal neurons synaptically target LHA orexin neurons, and that the LDTg is a likely 2nd-order targets of vHPC GHSR1a -> LHA orexin signaling.

Fluorescence in situ hybridization analyses revealed GHSR1a mRNA expression in the ventral CA1 field (vCA1), and further, that the vCA1 GHSR1a expression is predominantly isolated within the glutamatergic (VGLUT1-expressing) projection neurons of the pyramidal layer (Figure 3a). These pyramidal vCA1 glutamatergic neurons have previously been shown to densely innervate the LHA (19, 53), as well as the medial prefrontal cortex (18) and lateral septum (54, 55). To identify second-order targets of the vCA1 neurons that monosynaptically communicate to LHA, we utilized a dual-synaptic virus-based pathway tracing approach (Figure 3b) (16, 51). A unilateral iontophoretic co-injection of AAV2/1-hSyn-Cre and CTB-488 (imaged in AF647 channel to confirm injection site) targeted to the vCA1 induces Cre recombinase expression in neurons at the injection site (vCA1 HPC) and in first-order (but not second-order) neurons (Figure 3c, left) (16, 51). This was followed by a unilateral and ipsilateral pressure injection of AAV1-CAG-FLEX-TdTomato (a Cre-dependent anterograde tracer) targeted to the LHA (Figure 3d, right). Five animals were confirmed as double hits in both vCA1 HPC (Figure 3c) and LHA (Figure 3d) injection sites. In these rats, i.p. CCK-8 (3ug/kg) administration 90-min before tissue harvest induced c-Fos protein immunoreactivy in dorsal perifornical LHA (dpLHA) neurons that colocalize with Cre-expressing neurons (AAV-Flex-Tdtomato) receiving direct synaptic input from the vCA1 (Figure 3e). Quantitative analyses indicate i.p. CCK-8 significantly increased the percentage of AAV-Flex-Tdtomato cells that co-express c-Fos protein in the dpLHA relative to i.p. saline treatment (Figure 3f; $F_{[1,3]}$=4.6, p=0.007). Collectively, these findings indicate that CCK-8, a vagally-mediated satiation signal, engages dpLHA neurons that receive direct synaptic input from vCA1 glutamatergic neurons.

Given previous work identifying interactions between ghrelin and orexin signaling in promoting appetite (19, 56), we hypothesized that LHA orexin neurons receive input from the GHSR1a-expressing vCA1 glutamatergic neurons. Results from the five double hit animals reveal a population of LHA orexin immunoreactive neurons that are colocalized with Tdtomato (i.e., Cre-expressing neurons that receive vCA1 synaptic input) (Figure 4b),

and further, we identified Cre-expressing (Tdtomato+) putative axon terminal fields in the LDTg that overlap with orexin immunoreactive terminal fields (Figure 4c). Based on previous results demonstrating that GLP-1-producing neurons in the nucleus tractus solitarius (NTS) project to the LDTg to reduce food intake via a selective reduction in meal size (57), we next examined whether ORX-1R and GLP-1 receptor (GLP-1R) colocalize in LDTg neurons. FISH analyses identify substantial colocalization of ORX-1R and GLP-1R mRNA expression in the LDTg, with 59.6% of ORX-1R mRNA-positive cells containing GLP-1R mRNA (representative image; Figure 3e). Schematics of a representative double hit animal IHC and FISH analyses are displayed in Figures 3 and 4. Overall, these results indicate that GHSR1a is expressed in vCA1 glutamatergic projections neurons in the pyramidal layer, and that these neurons communicate monosynaptically to LHA orexin neurons that project to the LDTg.

Experiment 4: vHP ghrelin signaling increases meal size via downstream orexin signaling in the LDTg

Having identified LDTg ORX-1R signaling as a putative 2nd-order target of vHPC GHSR1a signaling, we next examined whether this pathway is relevant for vHPC ghrelin effects on meal size control. This was examined using a within-subjects neuropharmacological approach that utilized automated food intake monitors for analyses of spontaneous meal patterning in free-feeding rats. Results reveal that vHPC ghrelin administration just prior to the onset of the nocturnal feeding cycle increased 2hr cumulative food intake via a selective increase in meal size, and that this effect was blocked by LDTg ORX-1R antagonist administration (statistical results in Supplementary Results). Overall, these results demonstrate that vHPC ghrelin signaling promotes food intake by increasing spontaneous meal size, and that these effects require downstream ORX-1R signaling in the LDTg.

Discussion

Meal size control involves bidirectional communication between gastrointestinal-derived biological signals and the brain. Research on the how the brain regulates satiation processing has predominantly focused on the hypothalamus and the hindbrain (4), with data from decerebrate rats highlighting the sufficiency of hindbrain processing for critical aspects of meal size control (4, 58–60). However, meal size control is potently influenced by memory for recent eating occasions (61–64) and social factors (65) and therefore must undoubtedly involve processing in brain substrates that regulate higher-order cognitive functions. The ventral hippocampus (vHPC) has recently been linked with mnemonic control of feeding behavior (10, 66, 67). Here we expand this previous work and reveal that the stomach-derived endocrine signal ghrelin acts in the vHPC to counteract the intake-reducing effects of various gut-derived satiation signals, including CCK, Exendin-4, amylin, and mechanical distension of the stomach. Considering that amylin, unlike CCK, Exendin-4, and distension, acts to reduce food intake via non-vagal mechanisms (46), our results demonstrate that vHPC ghrelin signaling attenuates the efficacy of meal-derived satiation signals that communicate to the brain via multiple signaling pathways.

One mechanism through which vHPC ghrelin signaling can override satiation signaling is by producing a perceived internal state of energy depletion. For example, damage to the hippocampus is associated with interoceptive agnosia in human amnesic patients who will consume multiple consecutive meals without altering self-reported levels of hunger and fullness (68, 69). Similarly in rats, both selective HPC lesions (49) and consumption of a Western diet (70) impair the ability of interoceptive energy status cues to exert discriminative responding over conditioned appetitive behavior in the deprivation intensity discrimination task (DID). Ghrelin is a candidate signal for communicating an interoceptive state of energy restriction to hippocampal neurons, as both peripheral and intracerebroventricular ghrelin administration generalizes to a state of 24hr food restriction in the DID task (48). Indeed, results from the present study identify the vHPC as a site mediating these effects. Following vHPC ghrelin treatment, nonrestricted rats tested in the DID task demonstrated anticipatory appetitive responding consistent with their performance on 24hr-restricted training days, indicating that the treatment produced a perceived state of 24hr energy restriction. These results cannot simply be explained by vHPC increasing appetitive behavior generally speaking, as rats in Group 0+ *reduced* appetitive responding following vHPC ghrelin relative to vehicle treatment, an outcome mirroring their performance during training where internal cues arising from 24hrs food restriction were trained as a nonreinforced condition for this group.

It is also possible that ghrelin promotes an interoceptive perception of energy deficit through GHSR1a expressed on vagal afferent terminals, as capsaicin-induced elimination of vagal afferent signaling attenuates peripheral pharmacological ghrelin-induced hyperphagia (71). However, another study demonstrated that intraperitoneal administration of ghrelin stimulated eating in rats with subdiaphragmatic vagal deafferentation (72), suggesting that vagal signaling may not be required for the present results. Indeed, we recently demonstrated that the learned ability to use interoceptive hunger and satiety cues as discriminative stimuli for food reinforcement in the DID task is sustained following total subdiaphragmatic vagotomy (16). Therefore, present data revealing the ability of vHPC ghrelin signaling to provide an interoceptive “hunger-like” cue likely involves non-vagal mechanisms (e.g., BBB ghrelin transport from vasculature to vHPC), although this hypothesis requires further examination.

In the present study, vHPC ghrelin administration increased 2hr cumulative intake through a specific effect on meal size without influencing meal frequency. These findings are consistent with present data revealing that vHPC GHSR1a activation attenuates the efficacy of various satiation signals and produces an interceptive state similar to 24hr food restriction, as hyperphagia following periods of acute food restriction in rats is based predominantly on increased meal size and not frequency (73, 74). However, our data differ from previous work showing that activation of GHSR in the vHPC increases both meal size and meal frequency in rats (37), although we note that this discrepancy could be due to differences between testing animals during light (37) versus dark/nocturnal (present study) cycle. Taken together, previous work and present findings suggest that vHPC ghrelin signaling enhances meal size independent of light/dark cycle, whereas upregulating this system may only increase meal frequency at times in which animals are not normally feeding (light cycle).

To identify downstream neural pathways through which vHPC ghrelin signaling enhances meal size, we utilized a dual-synaptic virus-based pathway tracing strategy (51). The LHA was selected as a first-order target for Cre-dependent anterograde tracer administration as previous work established the dpLHA as a direct synaptic target from pyramidal projection neurons in vCA1 HPC (53, 75), and here we confirm that these pyramidal neurons express GHSR1a. Results revealed that dpLHA neurons that receive synaptic input from the vCA1 HPC are activated by peripheral CCK, and are predominantly orexin-producing neurons. While these data suggest that orexin neurons responsive to CCK also receive synaptic input from vCA1 neurons, this was not confirmed in the present study due to methodological limitations for co-labeling of orexin, TdTomato, and c-Fos signals. Previous literature has shown that in addition to feeding, orexin neurons in the dpLHA are involved in arousal and stress-related functions (76–80). Thus, it is possible that the capacity of vHPC ghrelin signaling to override gut-derived satiation signals and increase meal size is mediated, in part, by enhanced dpLHA orexin-mediated arousal or stress. However, this remains to be directly tested.

We further reveal that LHA orexin neurons that receive vCA1 input communicate downstream to the hindbrain LDTg, and that LDTg orexin signaling is required for vHPC ghrelin-induced elevations in spontaneous meal size. These findings complement other recent results identifying the LDTg as an emerging region of importance in food intake control. For example, the LDTg has many reciprocal projections with feeding-relevant forebrain and midbrain regions, including the LHA and the ventral tegmental nucleus (VTA) (81, 82). Further, LDTg neurons and glial cells express receptors for peptides that have established effects on food intake (such as amylin, orexin, ghrelin, GLP-1) (34, 57, 83, 84), and this brain region has been shown to modulate both homeostatic (energy deficit-based) and reward-based feeding behaviors (57, 81–83, 85–90). The LDTg also has afferent and efferent connections with local hindbrain nuclei involved in satiation processing and oral motor control, including the NTS, parabrachial nucleus, paramedian reticular formation (82, 90). The mechanisms through which LDTg neurons interact with these local hindbrain substrates to regulate meal size control remains poorly understood.

While anatomical studies have previously identified orexin neuron projections in the LDTg (91–93), to our knowledge the functional role of LDTg ORX-1R signaling to feeding behavior has not been previously explored. Present data show that orexin input to the LDTg plays a role in food intake and satiation signaling, as ORX-1R blockade in the LTDg eliminated the effects of vHPC ghrelin to enhance cumulative food intake via an increase in meal size. These results are consistent with recent work showing that CNS-ghrelin mediated hyperphagia involves downstream orexin signaling (40), an effect that is counteracted by activation of leptin receptor-expressing neurotensin neurons (41). Our fluorescence in situ hybridization analyses further identified substantial ORX-1R and GLP-1R mRNA colocalization in the LDTg. Given that gastric distension activates GLP-1-producing neurons in the NTS (94), and that LDTg GLP-1R activation reduces meal size in free-feeding rats (57), future work could explore whether LDTg ORX-1R signaling functionally interacts with GLP-1R signaling in the LDTg to regulate meal size.

Meal size control is potently influenced by the integration of various complex cognitive and mnemonic variables, including the external context/environment, interoceptive energy status cues, social factors, and previous learned experiences. The present results illuminate a novel descending forebrain-hindbrain circuitry through which such integration putatively occurs. Specifically, results provide evidence that vHPC ghrelin signaling increases meal size by counteracting the efficacy of various gut-derived satiation signals. Given that ghrelin levels are elevated not only during energy restriction but also in anticipation of eating (23, 95), we hypothesize that vHPC ghrelin signaling represents a key component of a cognitive override system that allows for the consumption of larger meals at times when eating is convenient and/or an extended period without eating is anticipated to follow the meal (e.g., habitual meals, meal entrainment models). Our additional findings further identify the downstream neural pathways mediating these effects, through which vCA1 GHSR1a-expressing neurons communicate to LHA orexin neurons that project to the LDTg (Figure 6). Collective results add to an emerging body of research identifying brain systems traditionally involved with memory and cognition in the control of feeding behavior

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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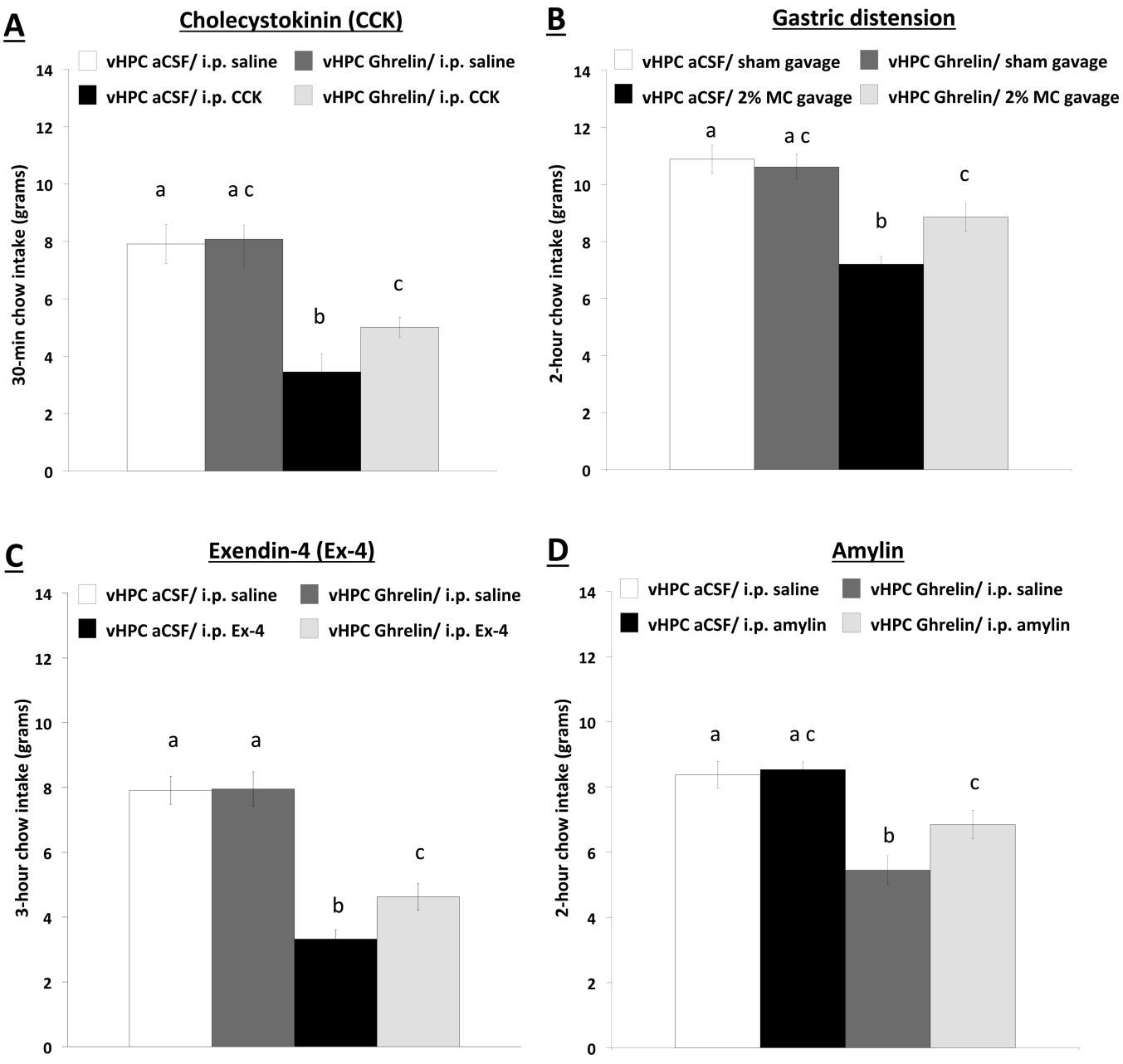

Figure 1.

Cumulative home cage chow intake in 24-hour food restricted rats at (a) 30-min following administration of a subthreshold dose of vHPC ghrelin (30pmol/200nl) and i.p. CCK (3 ug/kg) injections (n=9); at (b) 2-hour following vHPC ghrelin administration and gastric distension via 15 mL 2% MC oral gavage (n=13); at (c) 3-hour following vHPC ghrelin administration and i.p. Ex-4 (3 ug/kg) injections (n=20); and at (d) 2-hour following vHPC ghrelin administration and i.p. amylin (50 ug/kg) injections (n=23). Data are mean ± SEM; Means with different letters are statistically significant at $p<0.05$. vHPC: ventral hippocampus; aCSF: artificial cerebral spinal fluid; i.p.: intraperitoneal; CCK: cholecystokinin; MC: methylcellulose; Ex-4: Exendin-4.

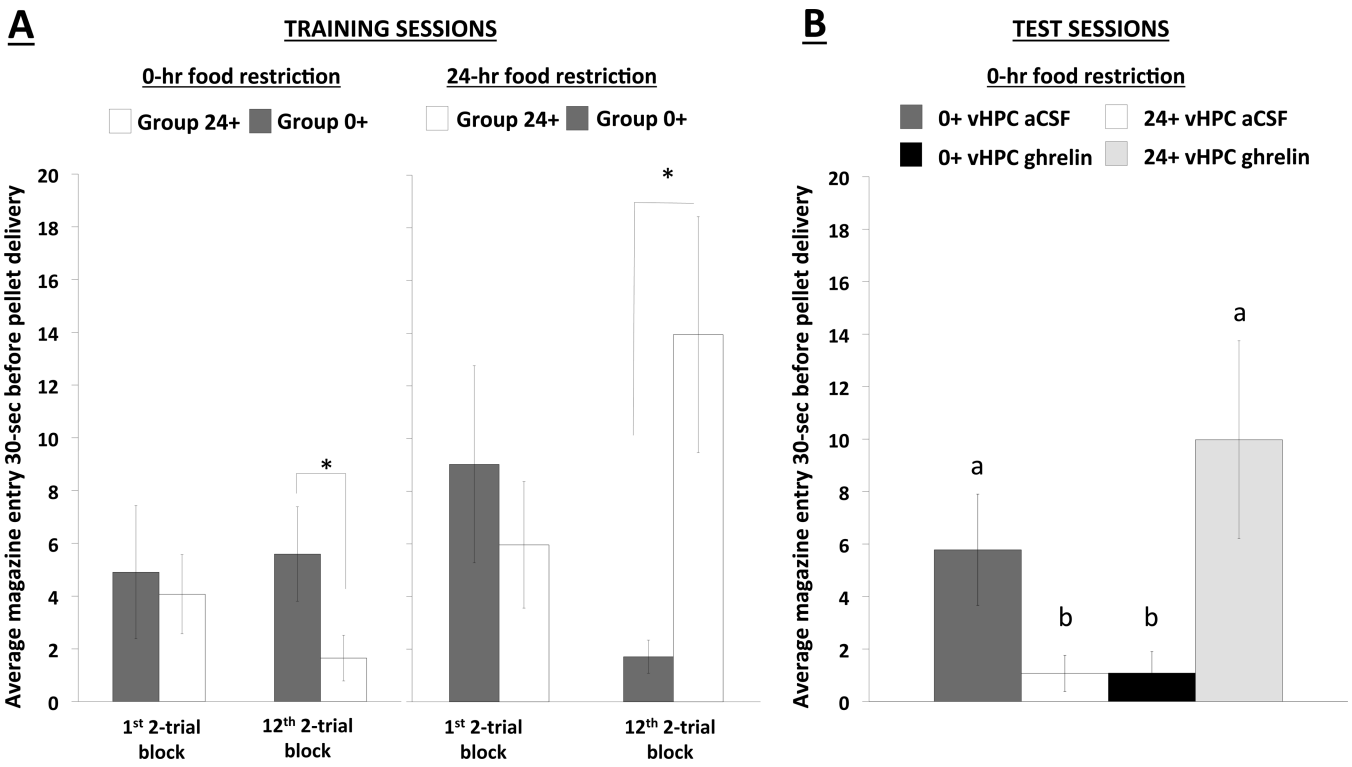

Figure 2.

(a) Mean magazine entries during the last 30-seconds before pellet delivery for the first and twelfth (last session before testing) two-trial blocks of deprivation intensity discrimination training sessions for Group 0+ (n=7) and 24+ (n=10) under alternating 0-hr (left panel) and 24-hr (right panel) food restriction. (b) Mean photobeam interruptions following vHPC ghrelin (150pmol/200nl) testing under 0-hr food restriction. Data are mean ± SEM; (a) *p<0.05 vs non-reinforced group. (b) Means with different letters are statistically significant at p<0.05. vHPC: ventral hippocampus; aCSF: artificial cerebral spinal fluid.

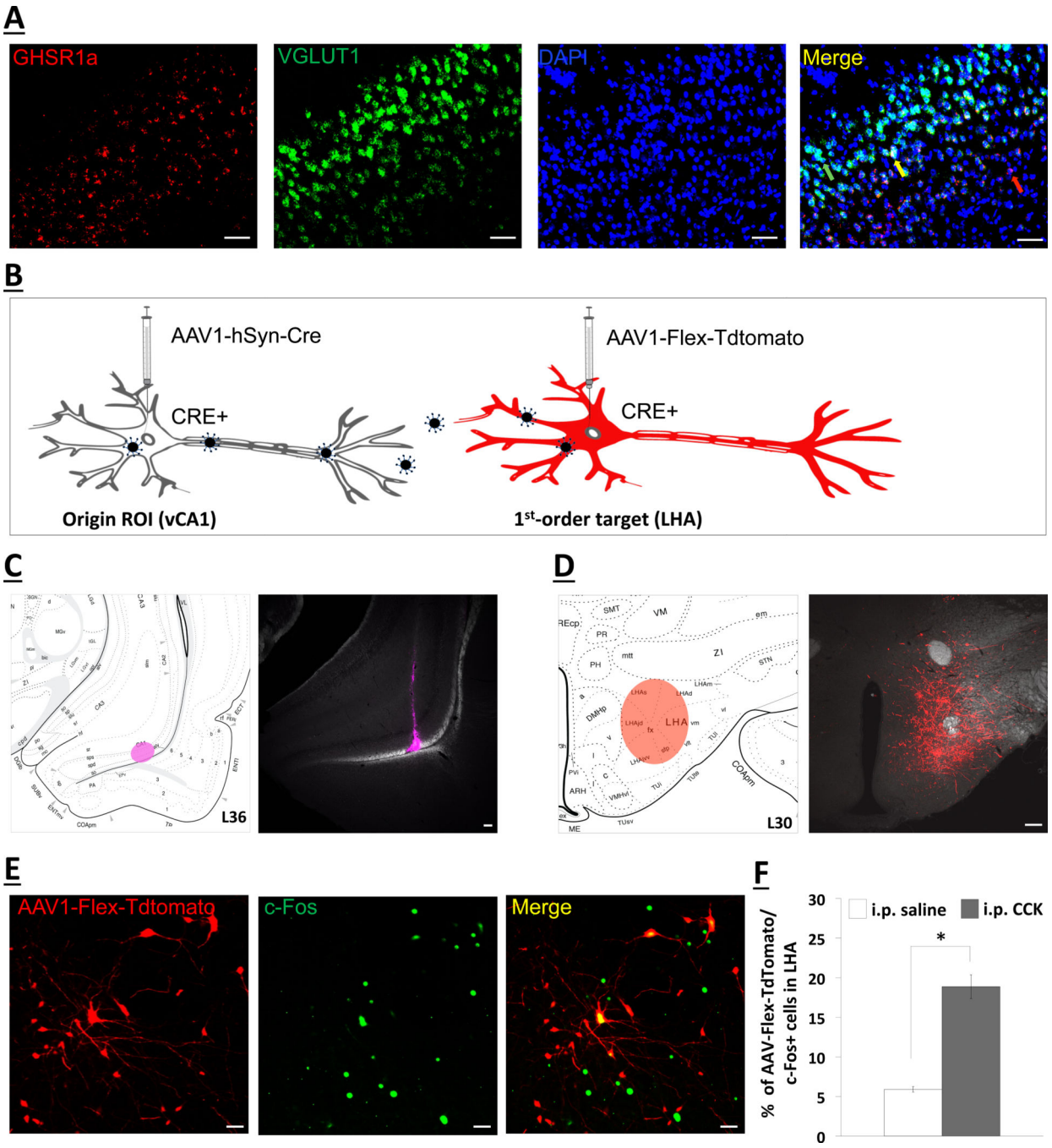

Figure 3.

(a) Representative images show GHSR1a mRNA (red) and VGLUT1 mRNA (green) expression in vCA1 cell bodies (DAPI nuclear stain; blue) (n=3). A representative red arrow depicts GHSR1a mRNA that is not vGLUT1; green arrow for vGLUT1 mRNA that is not GHSR1a; yellow arrow for vGLUT1/GHSR1a+ mRNA. Scale bar: 50μm. (b) Schematic illustration of Cre-mediated anterograde tracing method (51). The AAV2/1-hSyn-Cre drives Cre expression in origin region of interest (ROI) infected at the injection site (left), as well as in first-order (but not second-order) neurons based on virion release from first-order axon

terminals. The AAV-Flex-Tdtomato, a Cre-dependent anterograde tracer, will express red fluorescence in Cre+ neurons, including first-order neurons (right). (c) Right panel shows an ipsilateral and unilateral 200nl pressure vCA1 co-injection site of AAV-hSyn-Cre and CTB (far red) (n=5). Left panel shows schematic representative injection site in vCA1 HPC. (d) Right panel shows an ipsilateral and unilateral 200nl pressure injection site of a Cre-dependent anterograde tracer (red; AAV1-Flex-Tdtomato) in the lateral hypothalamic area (LHA). Left panel shows schematic representative injection site in LHA. Scale bar: 100μm. (e) Intraperitoneal injections of CCK (n=3) increases the percentage of AAV-Flex-TdTomato cells that colocalize with c-Fos positive immunoreactive cells in the LHA vs. saline (n=2). (f) Representative images of immunohistochemical staining of peripheral-CCK induced c-Fos protein (green) colocalize with neurons in the LHA that receive direct input from the vCA1 (red; AAV1-Flex-Tdtomato). Scale bar: 25μm. Data are mean 507 ± SEM; *$p<0.05$ vs saline treatment. i.p.: intraperitoneal; CCK: cholecystokinin; GHSR1a: type 1a growth hormone secretagogue receptor.

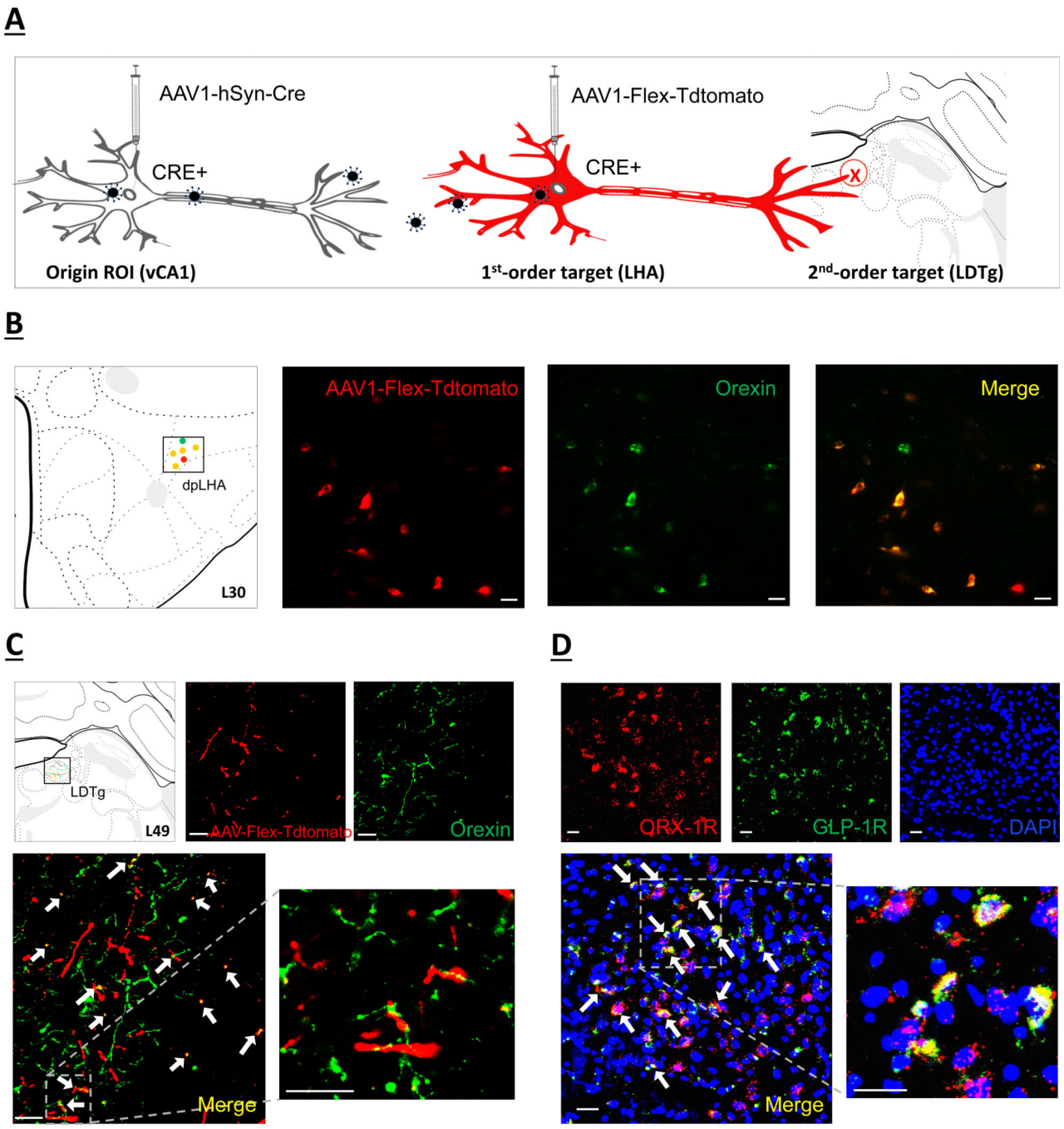

Figure 4.

(a) Schematic illustration of identifying second-order targets using a Cre-mediated multisynaptic anterograde tracing method (51). The AAV2/1-hSyn-Cre drives Cre expression in origin region of interest (ROI) infected at the injection site (left), as well as in first-order (but not second-order) neurons based on virion release from origin ROI axon terminals. The AAV-Flex-Tdtomato, a Cre-dependent anterograde tracer, will express red fluorescence in Cre+ neurons (center) and label axon terminals in second-order brain regions (right). (b) orexin immunofluorescent neurons (green) colocalize with neurons in the dpLHA

that receive direct synaptic input from the vCA1 (red; AAV1-Flex-Tdtomato). Scale bar: 25μm. Left panel shows schematic representation in Swanson Atlas level 30. (c) Overlap of second-order RFP-immunoreactive axon terminal fields in the LDTg (red) of LHA neurons that receive direct input from the vCA1 and orexin axon terminal fields (green). Arrows, co-expression of RFP and orexin terminal fields. Scale bar: 25μm. Left panel shows schematic representation of terminal field overlap in Swanson Atlas level 49. (d) Representative images show ORX-1R (red) GLP-1R (green) mRNA expression in LDTg cell bodies (DAPI nuclear stain; blue) (n=3). Arrows, co-expression of GLP-1R and ORX-1R mRNA cells. Scale bar: Scale bar: 25μm. dpLHA: dorsal perifornical region of the lateral hypothalamic area; LDTg: laterodorsal tegmental nucleus; ORX-1R: orexin-1 receptor; GLP-1R: glucagon-like peptide-1 receptor.

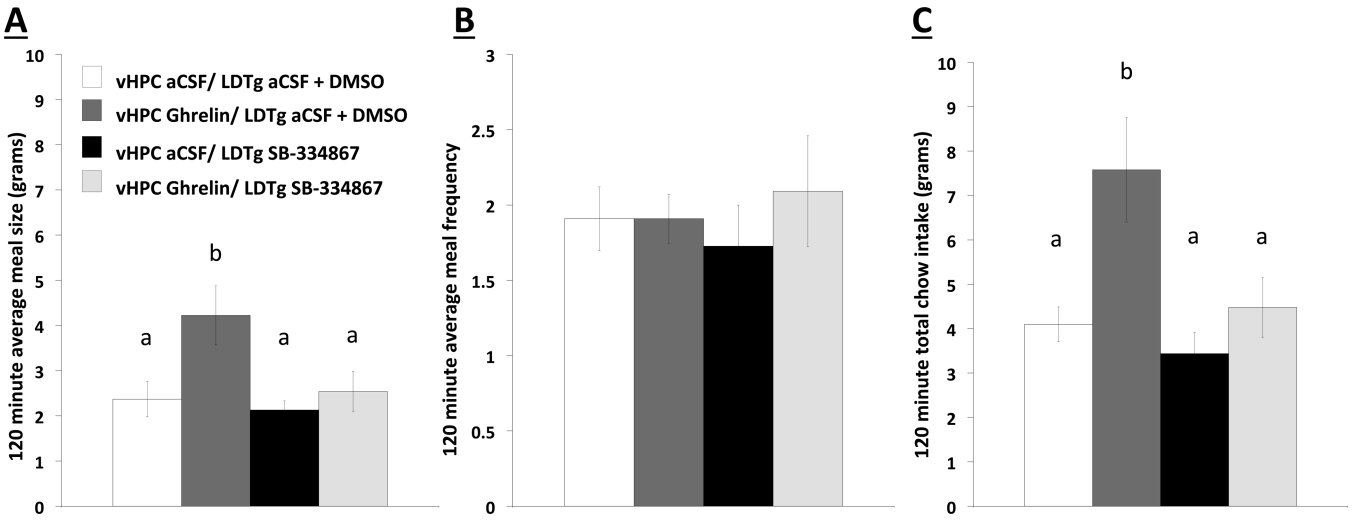

Figure 5.

Meal pattern analysis (n=11) following vHPC ghrelin (150pmol/200nl) and LDTg subthreshold dose of orexin 1-receptor antagonist (SB-334867; 1ug/200nl) administration: (a) 2 hr average meal size, (b) 2 hr average meal frequency, and (c) 2 hr total chow intake. Data are mean ± SEM; Means with different letters are statistically significant at $p<0.05$. LDTg: laterodorsal tegmental nucleus; DMSO: dimethyl sulfoxide.

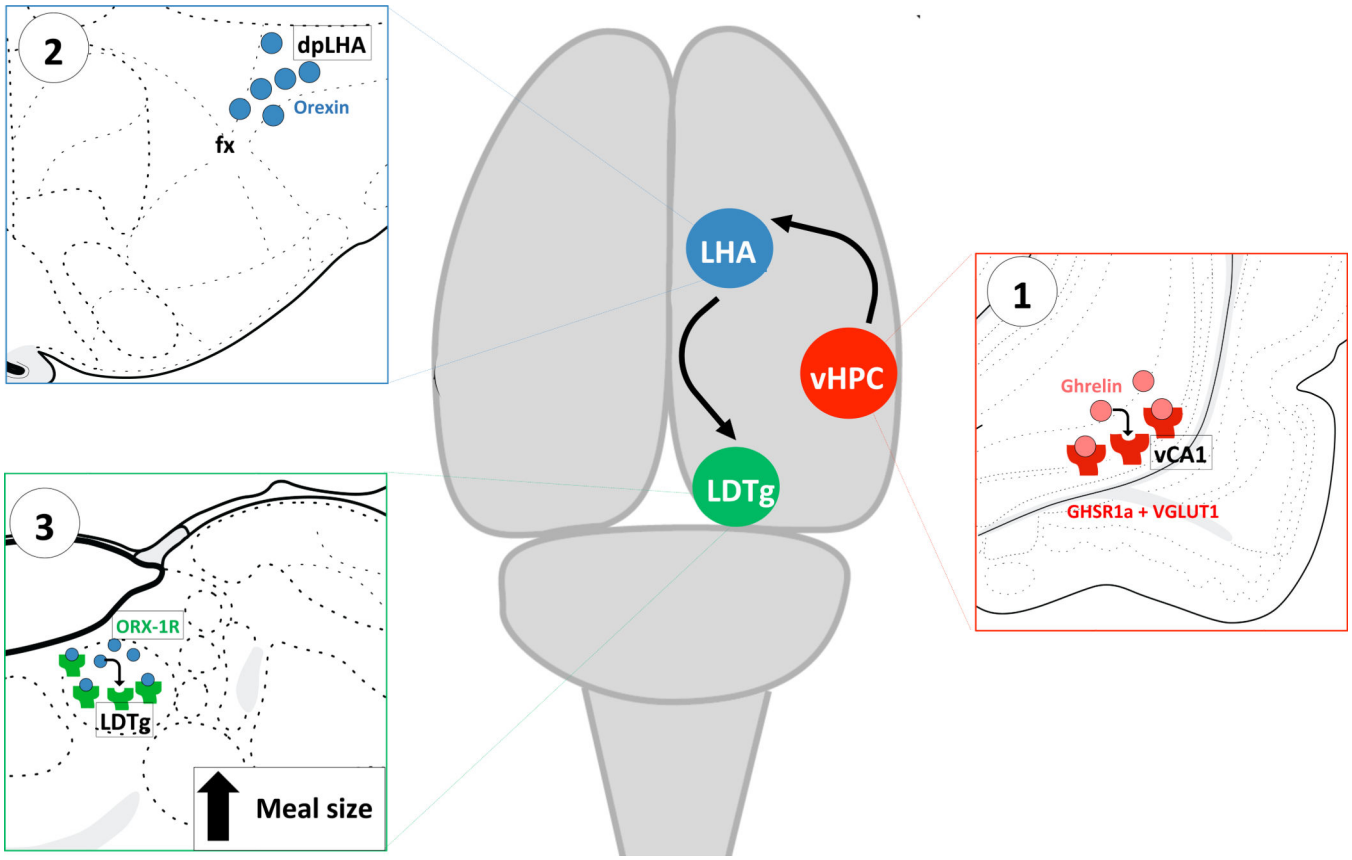

Figure 6.

Schematic illustration of the novel multi-order hippocampus-hypothalamic-hindbrain circuit. (1) GHSR1a is expressed in vCA1 glutamatergic projection neurons (red) in the pyramidal layer, which communicate monosynaptically to (2) dpLHA orexin neurons (blue) that project to (3) LDTg ORX-1R in the hindbrain (green). This pathway is critical for promoting meal size through interactions between vCA1 HPC ghrelin and downstream LHA orexin signaling to the LDTg. GHSR1a: type 1a growth hormone secretagogue receptor; dpLHA: dorsal perifornical region of the lateral hypothalamic area; LDTg: laterodorsal tegmental nucleus; ORX-1R: orexin-1 receptor.

KEY RESOURCES TABLE

Resource Type	Specific Reagent or Resource	Source or Reference	Identifiers	Additional Information
Add additional rows as needed for each resource type	Include species and sex when applicable.	Include name of manufacturer, company, repository, individual, or research lab. Include PMID or DOI for references; use “this paper” if new.	Include catalog numbers, stock numbers, database IDs or accession numbers, and/or RRIDs. RRIDs are highly encouraged; search for RRIDs at https://scicrunch.org/resources.	Include any additional information or notes if necessary.
Antibody	Rabbit anti-orexin	Phoenix Pharmaceuticals	RRIF: AB_10013632	
Antibody	Mouse anti-CTB	Abcam	RRID: AB 300499	
Antibody	Rabbit anti-RFP	Rockland	RRID: AB 2209751	
Antibody	Rabbit anti-cFos	Cell Signaling	RRID: AB_2106617	
Antibody	Donkey anti-mouse IgG-AlexaFluor 488	Jackson Immunoresearch	RRID: AB_2341099	
Antibody	Donkey anti-rabbit IgG-Cy3	Jackson Immunoresearch	RRID: AB_2307443	
Antibody	Donkey anti-goat IgG-AlexaFluor 750	Abcam	Cat #: ab175744	
Bacterial or Viral Strain	AAV1-hSyn-Cre-WPRE-hGH	Addgene	RRID: Addgene_105553	
Bacterial or Viral Strain	AAV1 -CAG-Flex-T dT omato-WPR E-rBG	Addgene	RRID: Addgene_51503	
Chemical Compound or Drug	Protease inhibitor cocktail	Sigma	Cat #: P8340–5ML	
Chemical Compound or Drug	Donkey serum	Jackson Immunoresearch	Cat #: 017–000-121	
Chemical Compound or Drug	Triton-X 100	Sigma	Cat #: X100–500ML	
Chemical Compound or Drug	Glycerol	EMD Millipore	Cat #: GX0185–5	
Chemical Compound or Drug	Ketaset (Ketamine), 100mg/mL	Henry Schein Animal Health	Cat #: 010177	
Chemical Compound or Drug	Anased (Xylazine) Injection, 100mg/mL	Henry Schein Animal Health	Cat #: 033198	
Chemical Compound or Drug	Aceproject (Acepromazine) Injection, 10mg/mL	Henry Schein Animal Health	Cat #: 003845	
Chemical Compound or Drug	Paraformaldehyde	Alfa Aesar	Cat # A11313	
Chemical Compound or Drug	Sodium hydroxide	EMD Millipore	Cat #: SX0590–1	
Chemical Compound or Drug	Sodium tetraborate	Alfa Aesar	Cat #: 40114	
Chemical Compound or Drug	Methylcellulose, 400 cP	Sigma-Aldrich	CAS #: 9004–67-5	
Chemical Compound or Drug	Tween-80	Sigma-Aldrich	CAS #: 9005–65-6	
Chemical Compound or Drug	EDTA Tetrasodium	EMD Millipore	Cat #: EX0550–5	

Resource Type	Specific Reagent or Resource	Source or Reference	Identifiers	Additional Information
Chemical Compound or Drug	Trizma hydrochloride	Sigma Aldrich	Cat #: T3252–250G	
Chemical Compound or Drug	Proteinase K	Sigma Aldrich	Cat #: P2308–100MG	
Chemical Compound or Drug	Triethanolamine	Sigma Aldrich	Cat #: T58300–1KG	
Chemical Compound or Drug	Acetic anhydride	EMD Millipore	Cat #: AX0080–6	
Chemical Compound or Drug	Citric acid trisodium	VWR	Cat #: 0101–500G	
Chemical Compound or Drug	ProLong Gold Antifade mounting medium	Cell Signaling	Cat #: 9071S	
Chemical Compound or Drug	aCSF	Harvard Apparatus	Cat #: 59–7316	
Chemical Compound or Drug	DMSO	MP Biomedicals, LLC	Cat #: 196055	
Commercial Assay Or Kit	GHSR1a probe	Advanced Cell Diagnostics	Cat #: 431991-C2	
Commercial Assay Or Kit	Glucagon-like peptide-1 receptor probe	Advanced Cell Diagnostics	Cat #: 315221-C2	
Commercial Assay Or Kit	hyocretin (orexin) receptor-1 probe	Advanced Cell Diagnostics	Cat #: 444761	
Commercial Assay Or Kit	vGLUT1 probe	Advanced Cell Diagnostics	Cat #: 317001-C2	
Commercial Assay Or Kit	RNAscope Fluorescent Multiplex Reagent Kit	Advanced Cell Diagnostics	Cat #: 320851	
Commercial Assay Or Kit	RNAscope Wash Buffer	Advanced Cell Diagnostics	Cat #: 320058	
Peptide, Recombinant Protein	Ghrelin	Bachem	Cat #: 4033076	
Peptide, Recombinant Protein	Cholecystokinin	Bachem	Cat #: 4003323	
Peptide, Recombinant Protein	Exendin-4	Bachem	Cat #: 4027457	
Peptide, Recombinant Protein	Amylin	Bachem	Cat #: 4030201	
Peptide, Recombinant Protein	SB-334867	Tocris Bioscience	Cat #: 1960	