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Hippocampus ghrelin receptor signaling promotes socially-mediated learned food preference

Ted M. Hsu [a, b], Emily E. Noble [a], David J. Reiner [c], Clarissa M. Liu [a, b], Andrea N. Suarez [a], Vaibhav R. Konanur [d], Matthew R. Hayes [c], Scott E. Kanoski [a, b, *]

[a] Human and Evolutionary Biology Section, Department of Biological Sciences, University of Southern California, Los Angeles, CA, USA
[b] Neuroscience Program, University of Southern California, Los Angeles, CA, USA
[c] Translational Neuroscience Program, Department of Psychiatry, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[d] Graduate Program in Neuroscience, University of Illinois at Chicago, Chicago, IL, USA

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ABSTRACT

Social cues are potent regulators of feeding behavior, yet the neurobiological mechanisms through which social cues influence food intake are poorly understood. Here we investigate the hypothesis that the appetite-promoting gut-derived hormone, ghrelin, signals in the hippocampus to promote learned social aspects of feeding behavior. We utilized a procedure known as 'social transmission of food preference' (STFP) in which rats ('Observers') experience a social interaction with another rat ('Demonstrators') that recently consumed flavored/scented chow. STFP learning in Observer rats is indicated by a significant preference for the Demonstrator paired flavor of chow vs. a novel unpaired flavor of chow in a subsequent consumption choice test. Our results show that relative to vehicle treatment, ghrelin targeted to the ventral CA1 subregion of the hippocampus (vHP) enhanced STFP learning in rats. Additionally, STFP was impaired following peripheral injections of L-cysteine that reduce circulating ghrelin levels, suggesting that vHP ghrelin-mediated effects on STFP require peripheral ghrelin release. Finally, the endogenous relevance of vHP ghrelin receptor (GHSR-1A) signaling in STFP is supported by our data showing that STFP learning was eliminated following targeted viral vector RNA interference-mediated knockdown of vHP GHSR-1A mRNA. Control experiments indicate that vHP ghrelin-mediated STFP effects are not secondary to altered social exploration and food intake, nor to altered food preference learning based on nonsocial olfactory cues. Overall these data reveal a novel neurobiological system that promotes conditioned, social aspects of feeding behavior.

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1. Introduction

Feeding is a complex and multifaceted behavior that often occurs in the absence of metabolic need. Decisions about feeding, such as when to eat, what to eat, and how much to eat, are strongly rooted in learned associations between environmental cues, interoceptive cues (e.g., hunger, satiety), and the sensory properties of specific foods (e.g., flavor, palatability) (Cornell et al., 1989; Davidson et al., 2014; Halford et al., 2004; Holland et al., 2002; Kanoski and Grill, 2017). Social cues, in particular, are potent environmental regulators of feeding behavior. For example, studies show that people often consume more food when in groups compared to eating alone, a phenomenon known as the "social facilitation of feeding" (Herman, 2015). Social cues can also reduce food consumption depending on social-derived norms and the context (Herman et al., 2003). In rodents, a behavioral paradigm known as the "Social Transmission of Food Preference" (STFP) has been widely used to examine social food-related learning. In this task learned associations formed between a socially-transmitted olfactory food cue (e.g. food odor detected from another animal) have a robust effect on an animal's subsequent food preferences (e.g. preference for the food associated with the odor smelled on the other animal's breath) (Brightwell et al., 2005; Countryman and Gold, 2007; Countryman et al., 2005; Galef et al., 2005; Galef and Whiskin, 2003; Gold et al., 2011; Hegde et al., 2016; Matta et al., 2017). Using a rodent model to better understand the neural

Abbreviations: STFP, Social transmission of food preference; vHP, ventral hippocampus; GHSR-1A, growth hormone secretagogue receptor-1A.

* Corresponding author. University of Southern California, 3616 Trousdale Parkway, AHF-252, Los Angeles, CA 90089-0372, USA.
E-mail address: kanoski@usc.edu (S.E. Kanoski).

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substrates that facilitate STFP and other learned aspects of feeding behavior is an important step towards elucidating the neurobiological pathways controlling higher-order aspects of food intake and body weight regulation.

The stomach-derived hormone ghrelin is emerging as a possible biological mechanism that links learning, memory, and ingestive behaviors (Cone et al., 2015; Hsu et al., 2016). Traditionally known as a "hunger" hormone, recent data suggest that ghrelin might be more aptly described as a conditioned, meal-anticipatory hormone. This notion is supported by findings that circulating ghrelin secretion can be entrained in both rodents and humans, resulting in conditioned cephalic responses through which ghrelin levels rise prior to an anticipated meal (Cummings et al., 2001; Drazen et al., 2006; Frecka and Mattes, 2008). Research has also demonstrated that ghrelin signaling modulates feeding in response to discrete conditioned food cues (e.g., lights, tones) (Dailey et al., 2016; Kanoski et al., 2013) and animals with transgenic ghrelin receptor (GHSR-1A) deletion show a lack of meal anticipatory behaviors in response to either circadian or environmental conditioned food cues (Blum et al., 2009; Davis et al., 2011; Lamont et al., 2014; Walker et al., 2012). Considering the important role of ghrelin signaling on learned feeding responses, it is feasible that ghrelin signaling in the brain might also regulate feeding behaviors stimulated by learned, social-related food cues.

Ghrelin readily crosses the blood brain barrier (Banks et al., 2002, 2008), and recent studies have identified central nervous system sites of action for ghrelin-mediated appetite and hyperphagia (Alvarez-Crespo et al., 2012; King et al., 2011; Schele et al., 2016; Skibicka et al., 2011, 2013; St-Onge et al., 2015). Among these various sites, the hippocampus, a brain region traditionally associated with learning and memory, has been identified as a site of importance in the higher-order control of feeding and energy balance (Benoit et al., 2010; Davidson et al., 2005, 2014; Kanoski, 2012; Kanoski and Grill, 2017; Parent et al., 2014; Tracy et al., 2001). Interestingly, ghrelin receptors are extensively expressed in the hippocampus, particularly in the ventral subregion (vHP) (Mani et al., 2014; Zigman et al., 2006). Work from our laboratory revealed that ghrelin acts in the vHP to modulate learned feeding behaviors in response to either environmental food-associated discrete stimuli (Kanoski et al., 2013) or circadian-driven food cues (Hsu et al., 2015). Importantly, social learning tasks like the STFP have been shown to be hippocampal-dependent (Carballo-Marquez et al., 2009; Countryman and Gold, 2007; Countryman et al., 2005; Winocur et al., 2001), and the vHP subregion has been identified as a critical neural substrate that regulates learning and memory processes related to social cues (Countryman et al., 2005; Gold et al., 2011; Hegde et al., 2016; Smith et al., 2007). Collectively, these data provide support for a hypothesis where ghrelin signaling in the vHP acts as an integrative central endocrine process that facilitates learned aspects of socially-driven feeding behaviors. The current manuscript utilizes multiple levels of analyses involving site-specific neuropharmacology, vHP targeted adeno-associated virus (AAV)-mediated GHSR-1A knockdown, and behavioral analyses to determine whether vHP ghrelin signaling plays an endogenous role in modulating learned feeding behaviors induced by social food-related cues.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Envigo, Indianapolis, IN; PND 60; 320–450 g) were individually housed in hanging wire cages in a temperature controlled vivarium with *ad libitum* access (except where noted) to water and food (LabDiet 5001, LabDiet, St. Louis, MO) on a 12 h:12 h reverse light/dark cycle. For all surgical procedures, rats were anesthetized via intramuscular ketamine 90 mg/kg and xylazine, 2.8 mg/kg and acepromazine, 0.72 mg/kg injections, followed by subcutaneous injections of analgesia (Ketoprofen, 5 mg/kg). All procedures were approved by the Institute of Animal Care and Use Committee at the University of Southern California.

2.2. Viral preparations and drug injections

2.2.1. Drug injections

For central drug injections into the vHP, guide cannulae (26-gauge, Plastics One, Roanoke, VA) were implanted and affixed to the skull with jewelers screws at the following stereotaxic coordinates (Paxinos, 2009): −4.9 mm anterior/posterior (AP), ±4.8 mm medial/lateral (ML), −6.1 mm dorsal/ventral (DV). Injectors for drug administration projected 2 mm beyond the guide cannulae. Experiments involving central drug injections included bilateral vHP (CA1 region) injections of ghrelin (Bachem, Torrance, CA), which was dissolved in artificial cerebral spinal fluid (aCSF). Injections were administered using a microinfusion pump (Harvard Apparatus, Holliston, MA) connected to a 33-gauge microsyringe injector through the indwelling guide cannulae. Flow rate was calibrated and set to 5 μl/min and 100 nl injection volume per hemisphere. Injectors were left in place for 30-sec to allow for complete infusion of the drug. Placements for vHP cannulae were verified post-mortem by injection of 100 nl blue dye (100 nl, 2% Chicago sky blue ink) through the guide cannulae. Data from animals with dye confined to the vHP were included in the analyses. In total 10% of rats with vHP cannulae were removed from the data because of misplaced cannulae.

To reduce peripheral circulating ghrelin levels, an amino acid compound, L-cysteine (Sigma-Aldrich) was used (McGavigan et al., 2015; Sirohi et al., 2017). L-cysteine was dissolved in 0.9% saline and delivered intraperitoneally (IP, 2 mmol/kg).

2.2.2. AAV-mediated RNA interference for GHSR-1A

For *in vivo* knockdown of GHSR-1A expression, short hairpin RNA targeting GHSR-1A mRNA was cloned and packaged into an adeno-associated virus (AAV2; Vector Biolabs) under the control of a U6 promoter and co-expressing green fluorescent protein (GFP) downstream of the U6 promoter (titer = 1.7e13 GC/ml). The shRNA sequence is: CCGG-ACTGCAACCTGGTGTCCTTTG-CTCGAG-CAAGGACACCAGGTTGCAG-TTTTTG. A scrambled shRNA, GFP-expressing AAV2 downstream of a U6 promoter (titer = 1.7e13 GC/ml) was used as a control (Vector Biolabs). AAVs were then delivered bilaterally to the vHP (AP: −4.9 ML: ±4.8 DV: −7.8) at an injection volume of 200 nl per hemisphere via pressure injection with the microinfusion pump setup described above.

Following all experimental procedures, animals were anesthetized, then transcardially perfused with ice-cold 0.9% saline, followed by 4% paraformaldehyde (PFA) in 0.1 M borate buffer (pH 9.5). Brains were removed and immersed in 12% sucrose in PFA fixative for 20–24 h at 4 °C. The brains were then flash-frozen in dry-ice cooled isopentane before sectioning on a sliding microtome at 30 μm. For histological verification, transverse sections of the vHP were slide mounted and viewed under a fluorescent microscope (Nikon 80i) until GFP-expressing cells were visualized in the vCA1. In separate cohort of animals with vHP control AAV (n = 7) or GHSR-1A KD (n = 8), bilateral micropunches (1 × 1x1 mm) were taken from this region and used for subsequent qPCR analyses. GHSR-1A mRNA levels were quantified using Taqman gene expression kits (GHSR-1A: Rn00821417; GapDH: Rn01775763_g1; Life Technologies) and PCR reagents (Applied Biosystems). qPCR

was conducted using an Eppendorf Mastercycler ep realplex2 and the comparative threshold cycle method was used to quantify relative mRNA expression. Overall 12% of rats were removed from analyses for lack of GFP expression or missed AAV placement.

2.3. Social transmission of food preference (STFP) task

To examine learned feeding based on social cues, we utilized the social transmission of food preference (STFP) task and adapted protocols from (Countryman and Gold, 2007; Countryman et al., 2005; Galef et al., 2005; Galef and Whiskin, 2003; Gold et al., 2011) (outlined in Fig. 1). First, untreated normal adult rats are designated as "Demonstrators", while experimental groups are designated as "Observers". Demonstrators and Observers are first habituated to a powdered rodent chow [LabDiet 5001 (ground pellets), LabDiet, St. Louis, MO] overnight. 24hr later, Observers are then individually assigned to demonstrators and are habituated to social interaction, where rat dyads are placed in a social interaction arena (23.5 cm W x 44.45 cm L x 27 cm H clear plastic bin with Sani-chip bedding) and allowed to interact for 30min. Both Observers and Demonstrators are returned to their homecages and food is withheld for 23hr prior to the social interaction. For the social interaction, in a room separate from Observers, Demonstrators are given the opportunity to consume one of two flavors of powdered chow (flavored with 1% cinnamon or 2% cocoa, 2% marjoram or 0.5% thyme; counterbalanced according to group assignments) for 30min. Our pilot studies and previous published work (Galef et al., 2005; Galef and Whiskin, 2003) show that rats equally prefer these flavors of chow. The Demonstrator rat is then placed in the social interaction arena with the Observer rat and allowed to socially interact for 30min. Observers are then returned to their homecage and allowed to eat *ad libitum* for 1hr. 23hr later, 23hr food-deprived Observer animals are given a home cage food choice test for either the flavor of chow paired with the Demonstrator animal or a novel, unpaired flavor of chow that is a flavor that was not given to the Demonstrator animal (1% cinnamon vs. 2% cocoa or 2% marjoram vs. 0.5% thyme; counterbalanced according to group assignments). 1hr food intake is recorded with spillage accounted for by weighing crumbs collected from Techboard paper that is placed under the cages of each animal prior to feeding. The % preference for the paired flavor is calculated as: 100*Demonstrator-paired flavored chow intake/Demonstrator + Novel flavored chow intake. In this procedure, normal untreated animals learn to prefer the Demonstrator paired flavor based on social interaction and smelling the breath of the Demonstrator rat (Countryman and Gold, 2007; Countryman et al., 2005; Galef et al., 2005; Galef and Whiskin, 2003; Gold et al., 2011).

2.4. Experiment 1: the effects of vHP GHSR-1A activation on STFP

To determine the effects of vHP GHSR-1A activation on STFP, Observer animals first received bilateral cannulae targeting the vHP as described above. After recovery, Observers underwent STFP procedures as described. First, Observers rats received bilateral vHP administration of ghrelin (n = 8, 150 pmol) or aCSF vehicle (n = 10) 60min prior to social interaction with untreated Demonstrator rats (n = 9; 1 Demonstrator rat assigned to 2 Observer rats, counterbalanced for experimental group) in a counterbalanced fashion. The

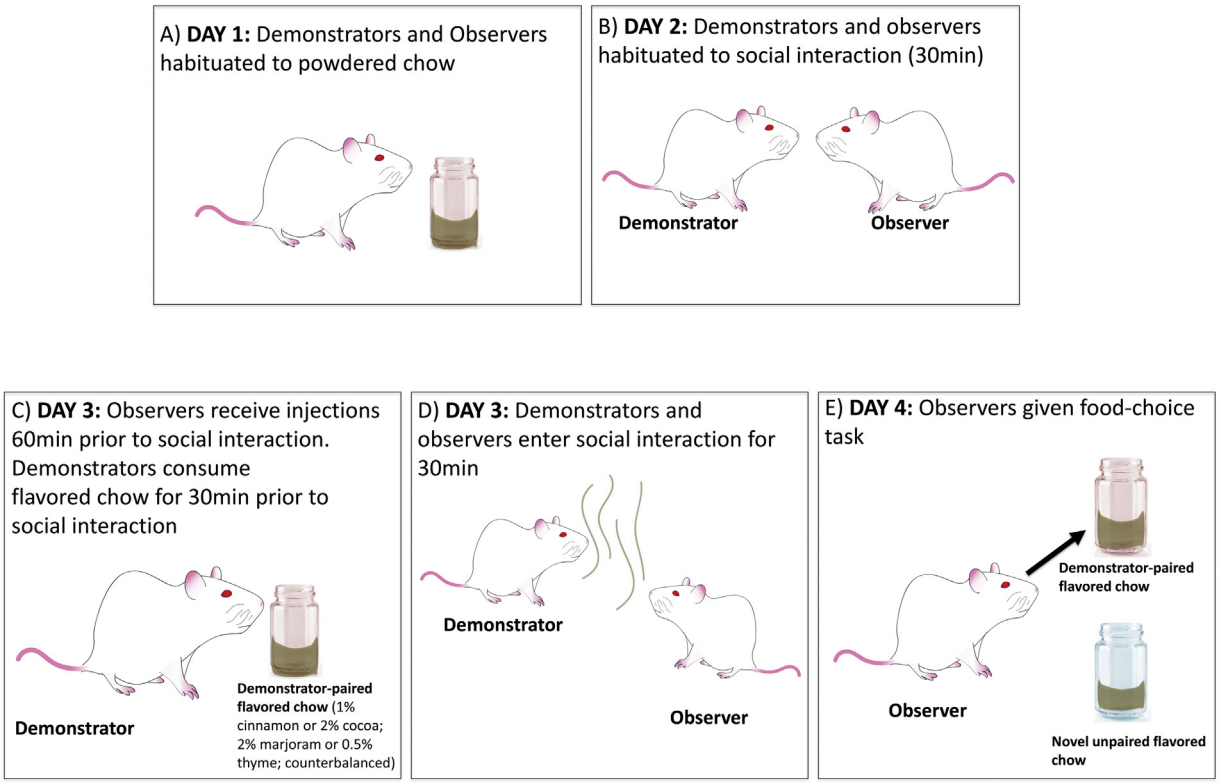

Fig. 1. A schematic outlining the Social Transmission of Food Preference Task (STFP). A) Day 1: Animals are designated as "Demonstrators" or "Observers" and habituated to powdered chow overnight. B) Day 2: Demonstrator and Observer pairs are habituated to social interaction in an arena separate from their home cages. C) Day 3: Demonstrator rats consume powdered chow that is flavored with particular spices (1% cinnamon or 2% cocoa; 2% marjoram or 0.5% thyme; counterbalanced) for 30min. D) Demonstrator animals are then moved to the arena where they are allowed to socially interact with Observer animals for 30min. E) 24hr later, Observers are given a food-choice task in their home cage where they are allowed to consume either the flavor of chow paired with the Demonstrator, or a novel unpaired flavor of chow (a flavor that the Demonstrator did not consume on Day 3). Normal, untreated control Observer animals typically prefer the Demonstrator-paired flavor of chow.

dose for ghrelin injections were based on (Kanoski et al., 2013) and chosen for its stimulatory effects on feeding behavior. During this time, social interaction is video recorded and an experimenter blind to experimental groups records the time spent in social interaction. Social interaction measures are adapted from (Segatto et al., 2014) and include time spent in the following activities: time spent pouncing, pinning, and sniffing the partner rat. 24hr after social interaction, Observers receive the preference consumption test as described above.

2.5. Experiment 2: involvement of peripheral ghrelin circulation in STFP

To determine whether peripherally-released circulating ghrelin is required for STFP learning, we utilized an amino acid compound, L-cysteine, which when delivered IP reduces peripheral ghrelin levels by ~50% (McGavigan et al., 2015; Sirohi et al., 2017) without affecting circulating GLP-1 and PYY. Using the same STFP task as described above, Observers received IP L-cysteine (n = 9, 2 mmol/kg) or 0.9% saline (n = 9) 30min before social interaction with Demonstrator rats (Demonstrator n = 9; 1 Demonstrator rat assigned to 2 Observer rats, counterbalanced for experimental group). 24hr later, a food choice test for the Demonstrator-paired flavored chow or novel flavored chow was administered as described above.

2.6. Experiment 3: effect of L-cysteine on total and active circulating ghrelin levels

To validate L-cysteine as an approach to reduce peripheral circulating ghrelin, we delivered L-cysteine (2 mmol/kg, n = 8) or saline (n = 7) IP to 23hr food deprived animals. 30min later, animals were decapitated under anesthesia and trunk blood was collected into tubes preloaded with 1 μl protease inhibitor cocktail (P8340, Sigma-Aldrich) and centrifuged at 15000 rpm for 10min to isolate serum (placed immediately in dry ice and then stored at −80 Celsius until analyses). Concentrations for active GLP-1, total ghrelin, and active ghrelin were detected using an enzyme-linked immunosorbent assays [ELISA; Millipore EZGLPHS-35K (GLP1), EZRGRT-91K (Total ghrelin), and EZRGRA-90K (Active ghrelin)] following the manufacturer's protocols.

2.7. Experiment 4: effects of vHP GHSR-1A KD on energy balance

Animals (n = 21) received one of the following 200 nl, bilateral injections to the vHP as described above: AAV-GHSR-1A shRNA (referred to as vHP GHSR-1A KD group; n = 9) or control AAV (referred to as CTRL AAV; n = 12). After surgical procedures, all animals were maintained on normal chow for 4 weeks and subsequently switched to a Western Diet (45% kcal from fat, enriched with sucrose; Research Diets D12451) for 3 weeks. Food intake (spillage accounted for) and body weight were tracked daily throughout this period.

2.8. Experiment 5: effects of vHP GHSR-1A KD on the social transmission of food preference

To determine the impact of vHP GHSR-1A KD on STFP, we used the same cohort of animals as Experiment 3 where vHP GHSR-1A KD (n = 9) or CTRL AAV (n = 12) animals are designated as Observers and underwent the STFP task (Demonstrator n = 12; 1 Demonstrator rat assigned to 2 Observer rats, counterbalanced for experimental group) as described above. Animals underwent STFP training 8 weeks following AAV surgeries. Following the STFP task, animals were placed back on chow diet.

2.9. Experiment 6: effects of vHP GHSR-1A KD on non-social olfactory transmission of food preference

To determine whether vHP GHSR-1A KD impairs olfactory processing and/or olfactory habituation learning to nonsocial cues, we modified the STFP paradigm with the social interaction component removed, termed the “Non-social olfactory transmission of food preference”. Importantly, this experiment involves a procedure that is identical to the STFP task, however there is no social interaction or Demonstrator rat involved. Briefly, 1 week following the STFP task, vHP GHSR-1A KD (n = 9) or control animals (n = 12) are first habituated to powdered chow. Under 23hr food deprivation, animals are placed in the same social interaction arena as above for 30min, however the Demonstrator rat is omitted. Instead, flavorings used in the STFP task (1% cinnamon or 2% cocoa; 2% marjoram or 0.5% thyme; counterbalanced) are mixed with the Sani-chip bedding placed in the arena. Observer animals are then returned to their homecage and allowed *ad libitum* access to chow for 1hr. 24hr later, a food choice test is given analogous to the STFP task.

2.10. Statistical analysis

Statistical analyses for group differences employed one-way analysis of variance (ANOVA) with Group as a between-subjects variable. Repeated measures ANOVA was also used to determine the presence vs. absence of significant STFP learning analyzed separately within groups. The α level for significance was 0.05. Statistical analyses were conducted with computer software (Statistica V7; Statsoft).

3. Results

3.1. vHP GHSR-1A activation increases STFP learning

Fig. 2A depicts a representative vHP injection site (Fig. 2B, corresponding schematic of injection site at Swanson atlas level 36). Results reveal that vehicle-injected, control rats are able to successfully learn the STFP paradigm, as reflected by a significant preference for the Demonstrator-paired flavor of chow during the food choice task administered 24hr after social interaction compared to baseline ($p < 0.05$). Observer animals who received vHP ghrelin injections prior to the social interaction phase also showed a significantly increased preference for the Demonstrator-paired flavor of chow vs. the nonpaired flavor of chow 24hr later in the food choice test ($p < 0.05$). Importantly, a group comparison for the percentage ratio for the paired vs. the unpaired flavor of chow confirmed that STFP was significantly elevated by vHP ghrelin relative to vehicle treatment (Fig. 2C; significant main effect of drug, $p < 0.05$ vs. vehicle). Total food intake during the food choice test, however, was not influenced by drug treatment during the conditioning phase (Fig. 2C, $p > 0.05$). While food intake was not recorded during the 1hr refeeding phase after the social interaction, we confirmed in an additional cohort of rats (n = 16) that this dose of vHP ghrelin has no significant effect on 1hr food intake following a 23hr fast (7.81 g consumed following vHP aCSF treatment [SEM = 0.55] and 8.36 g consumed following vHP ghrelin treatment [SEM = 0.35]). Furthermore, data obtained during the social interaction phase of the STFP task showed that vHP ghrelin treated animals spent a comparable amount of time in social interaction compared to vehicle treated rats (Fig. 2C). Since the 24hr food choice task occurs well after the typical hyperphagic effects of vHP GHSR-1A activation (typically up to 5 h, see (Hsu et al., 2015; Kanoski et al., 2013)) and we saw no effects of vHP GHSR-1A activation on social behaviors during the interaction, these data suggest that vHP ghrelin signaling promotes conditioned socially-

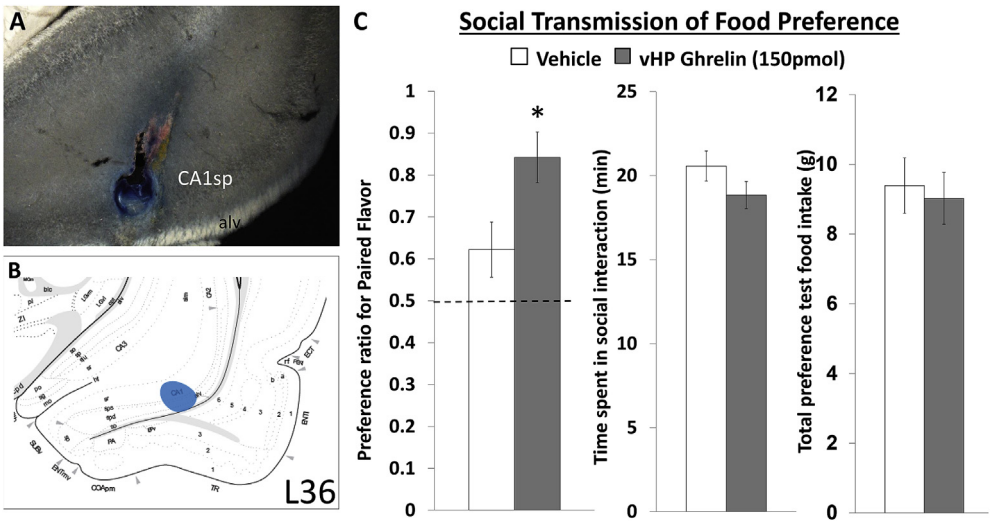

Fig. 2. A) Depicts a representative vHP injection site with a 100 nl Chicago sky blue ink injection. B) Corresponding schematic of vHP injection site at Swanson atlas level 36. C) % Preference for the Demonstrator paired flavor and total food intake in vehicle or vHP ghrelin (150 pmol, administered 60min before social interaction)-treated animals during the food-choice task 24hr after social interaction and time spent in social interaction during the interaction phase of STFP (24hr prior to food-choice task). *$p < 0.05$ vs vehicle. Data are mean ± SEM. CA1sp: CA1 pyramidal cells; alv = alveus.

mediated feeding behaviors.

3.2. Reduction of peripheral ghrelin levels impairs STFP

We sought to determine whether peripheral ghrelin circulation affects STFP. As stated in the Methods, IP administration of L-cysteine is capable of selectively reducing peripheral ghrelin levels by ~50% (McGavigan et al., 2015; Sirohi et al., 2017). Using the same STFP procedure as described above, Observers first received IP L-cysteine or 0.9% saline vehicle 30min before the social interaction phase of the task. Results demonstrate that in the food preference test 24hr later, well beyond the hypophagic effects of L-cysteine (up to ~1hr), vehicle treated animals appropriately learned the STFP task as reflected by a significant preference for the Demonstrator-paired flavor ($p < 0.05$). In contrast, IP L-cysteine administration prior to social interaction blocked the preference for the Demonstrator-paired flavor ($p > 0.05$). Importantly, STFP was significantly reduced by IP L-cysteine relative to vehicle treatment, demonstrated from a group comparison for the percentage ratio for the paired vs. the unpaired flavor chow consumption (Fig. 3, significant main effect of drug, $p < 0.05$ vs. vehicle), whereas IP L-cysteine had no effect on social behavior or total food intake during the food choice task relative to vehicle treatment (Fig. 3, $p > 0.05$). Moreover, this dose of L-cysteine had no significant effect on 1hr chow intake during the refeeding period following the social interaction (5.47 g consumed following IP vehicle treatment [SEM = 0.42] and 6.35 g consumed following IP L-cysteine [SEM = 0.40]). McGavigan et al. (2015) demonstrated that IP L-cysteine can reduce food intake during the 0–1hr period immediately following injection. We did not observe a similar reduction in food intake following IP L-cysteine treatment, perhaps due to the timing of when we measured food intake (between 1–2 hrs post injection).

Finally, in a separate cohort of rats, we validated the usage of L-cysteine as a method to reduce peripheral circulating ghrelin levels. Our data show that IP L-cysteine treatment significantly reduced both circulating total and active ghrelin levels relative to vehicle treatment (Fig. 4A and B respectively; $p < 0.05$ vs. vehicle) and had no effect on active GLP-1 levels (a gut-derived endocrine signal that, like ghrelin, influences hippocampal-dependent memory function (During et al., 2003); Fig. 4C).

3.3. Chronic vHP GHSR-1A KD impairs STFP independent of body weight or food intake

To examine the endogenous relevance of vHP ghrelin signaling on learned feeding behaviors, animals received either vHP injections of AAV2-GHSR-1A shRNA or scrambled control AAV2 as described above. After experimental procedures (~60 days), histological analyses confirmed successful AAV2-GHSR-1A shRNA transfection of vHP pyramidal neurons (Fig. 5A). Only animals with AAV spread confined to the vCA1 were included in the analyses (see Fig. 5B for a representative schematic of vHP AAV2-GHSR-1A shRNA GFP distribution). Consistent with previously published work utilizing this AAV2 GHSR-1A knockdown approach (Lopez-Ferreras et al., 2017), qPCR in AAV2 GHSR-1A shRNA infected micropunched vHP tissue showed a significant 50% knockdown of GHSR-1A mRNA compared to CTRL AAV (Fig. 5C, $p < 0.05$). We did not observe differences in standard rodent chow intake over a 4-week time period (data not shown, $p > 0.05$). Over the time course of all experiments there were no group differences in body weight gain (~12 weeks total; Fig. 5D, $p > 0.05$). Moreover, when animals were switched to a Western diet high in sugar and fat for 3 weeks, no differences in food intake were observed (Fig. 5E, $p > 0.05$).

To determine the endogenous relevance of vHP ghrelin signaling on learned socially mediated feeding behaviors, vHP GHSR-1A KD or CTRL AAV animals underwent the STFP task. Consistent with Experiment 1, CTRL AAV animals successfully learned the STFP task, as indicated by a significant preference for the Demonstrator-paired flavor in the food-choice test ($p < 0.05$). Comparatively, vHP GHSR-1A KD blocked the preference for the Demonstrator-paired flavor compared to the CTRL AAV group ($p < 0.05$). A group comparison for the percentage ratio for consumption of the paired vs. the unpaired flavored chow indicated a significant group difference (Fig. 6A, $p < 0.05$ vs CTRL AAV), whereas no group differences were observed in total food intake during the food choice task (Fig. 6A, $p > 0.05$). To account for a potential confound where vHP GHSR-1A KD might impact olfactory processing, we repeated the STFP task with the Demonstrator and social component

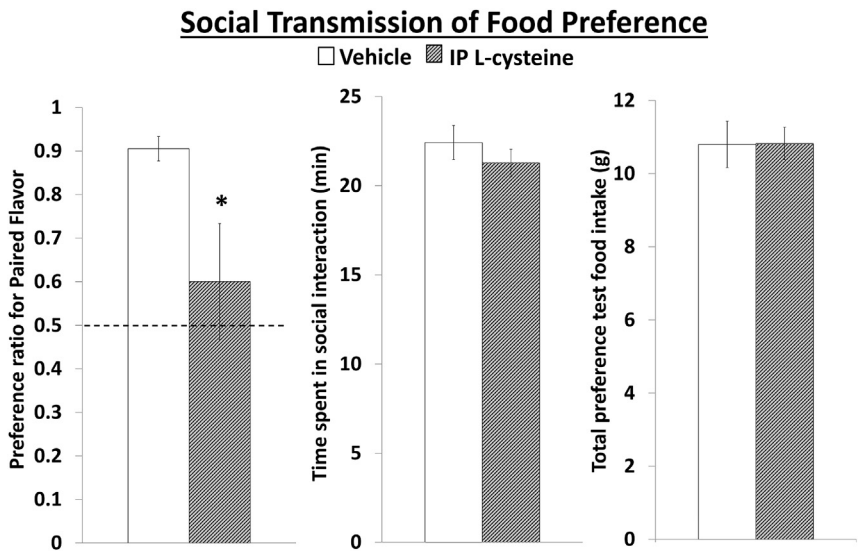

Fig. 3. % Preference for the Demonstrator-paired flavored chow, time spent in social interaction during the interaction phase of STFP, and total food intake during the food-choice test 24hr after social interaction during the STFP task in IP L-cysteine (2 mmol/kg) or vehicle treated rats (administered 30min before social interaction). *p < 0.05 vs vehicle. Data are mean ± SEM.

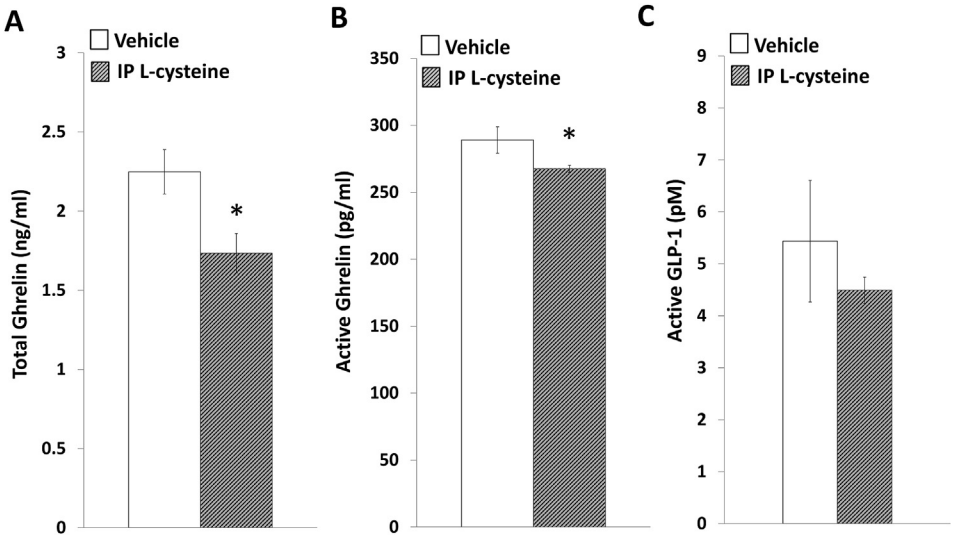

Fig. 4. A) Circulating levels of A) total ghrelin, B) active ghrelin, and C) active GLP-1 following IP administration of L-cysteine. *p < 0.05 vs. vehicle. Data are mean ± SEM.

removed ("Non-social olfactory transmission of food preference", see above). Results demonstrate that both vHP GHSR-1A KD and CTRL AAV animals comparably preferred the flavor of chow that was paired with the bedding (percentage preference depicted in Fig. 6B, p > 0.05), suggesting that vHP GHSR-1A KD selectively impairs learned, social transmission of food preference without deficits in olfactory processing or habituation learning.

4. Discussion

Learning and memory processes have a powerful influence on food intake and energy balance. Recent research has identified ghrelin as a particularly important biological substrate that modulates these types of conditioned, learned feeding behaviors (Hsu et al., 2016). Here we illuminate a novel neural substrate through which ventral hippocampus (vHP) neurons are integrated with ghrelin receptor signaling to facilitate socially-mediated food preferences. We first demonstrated that pharmacological activation of vHP GHSR-1As results in an increased preference for foods that are associated with social interaction with another rat. Importantly, while the animals had elevated vHP ghrelin signaling during the social interaction phase, the preference test occurred 24hr after vHP ghrelin administration, well beyond the typical hyperphagic effects of vHP GHSR-1A activation (up to 5 h, see (Hsu et al., 2015; Kanoski et al., 2013)). Importantly there were no group differences in the time spent during social interaction, indicating that vHP ghrelin did not interfere with general social exploration. Taken together, these data suggest that vHP GHSR-1A activation facilitates the learned aspects of socially-driven feeding behavior.

It should be noted that the current study did not systematically characterize the mnemonic constructs involved in vHP ghrelin mediated social food learning (e.g. acquisition, consolidation, retrieval). Recent work has demonstrated that vHP processing mediates the acquisition and consolidation of social long-term

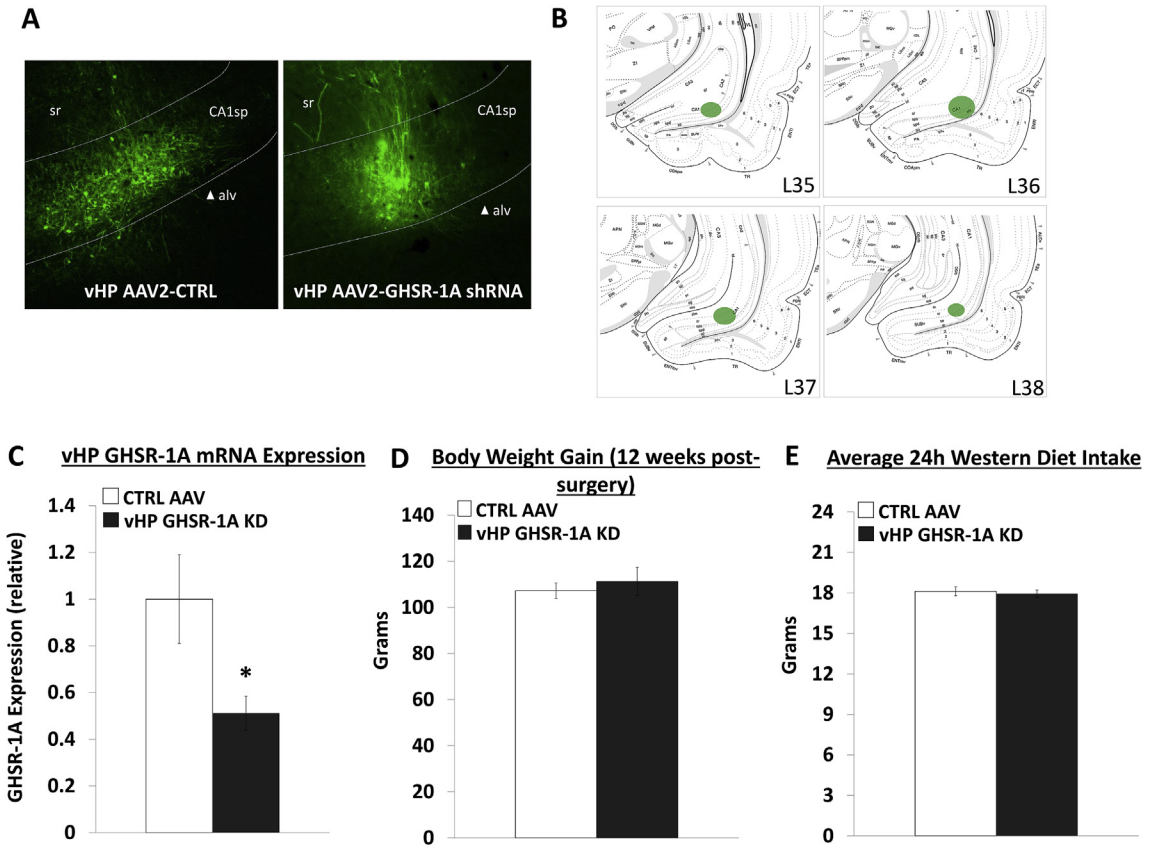

Fig. 5. A) vHP targeted AAV-GHSR-1A shRNA and scrambled shRNA control AAV transfects vHP neurons. B) Representative schematic of AAV-GHSR-1A shRNA injection spread through the rostral-caudal axis of the vHP (Swanson atlas levels 35–38). C) vHP AAV-GHSR-1A shRNA significantly reduces GHSR-1A mRNA expression by ~50%. D) Body weight gain over a 12-week post-surgery period in CTRL AAV and E) average 24hr Western diet intake beginning 4 weeks post-surgery and maintained for 3 weeks. *$p < 0.05$ vs CTRL AAV. Data are mean ± SEM. sr: stratum raditum; CA1sp: CA1 pyramidal cells; alv = alveus.

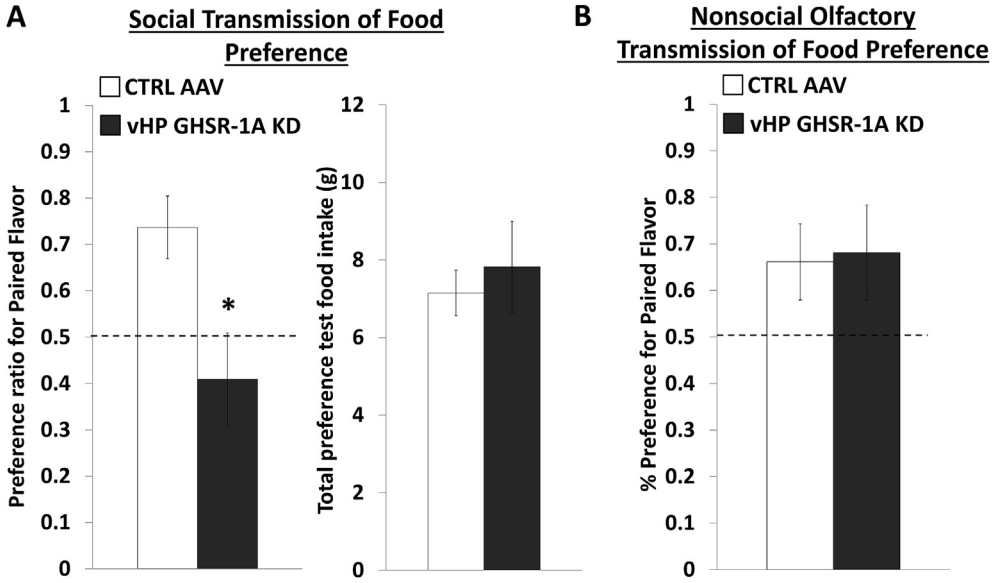

Fig. 6. A) % Preference for the Demonstrator-paired flavored chow and total food intake during the food-choice test 24hr after social interaction during the STFP task in vHP GHSR-1A KD or CTRL AAV animals. B) % Preference for the bedding-paired flavored chow in the food-choice test 24hr after exposure to olfactory cues during the Nonsocial Olfactory Transmission of Food Preference task. *$p < 0.05$ vs vehicle. Data are mean ± SEM.

memories (Countryman et al., 2005; Gold et al., 2011; Hegde et al., 2016; Smith et al., 2007). In the present study vHP ghrelin was administered prior to the conditioning phase (the social interaction) and therefore may have influenced acquisition and/or consolidation. Future studies could dissociate between ghrelin's effect on these three phases of memory formation and expression

by administering ghrelin immediately after the social interaction, or shortly before the retention/test phase.

GHSR-1A's have high levels of constitutive activity and recent investigations show that constitutively active GHSR-1As in the absence of ligand (ghrelin) binding can have effects on feeding and energy balance (Mear et al., 2013; Petersen et al., 2009). Furthermore, GHSR-1As are capable of dimerizing with other feeding-relevant receptor types (e.g. Type-1 dopamine receptors and melanocortin-3 receptors) (Jiang et al., 2006; Rediger et al., 2011; Wellman and Abizaid, 2015). Thus, there is the possibility that vHP GHSR-1A mediated STFP might be regulated by GHSR-1A constitutive activity and/or signaling mechanisms relating to GHSR-1A dimerization as opposed to peripheral ghrelin secretion and eventual binding to vHP GHSR-1As. In light of this, we examined the requirement of peripheral ghrelin circulation on the expression of STFP by reducing peripheral ghrelin levels with L-cysteine (McGavigan et al., 2015; Sirohi et al., 2017). In the current study, our results demonstrated that IP administration of L-cysteine prior to social interaction impairs STFP and reduces circulating total and active ghrelin levels without impacting active GLP-1 levels, suggesting that peripheral ghrelin secretion and central ghrelin ligand binding is necessary for GHSR-1A-mediated effects on learned social feeding behaviors. Consistent with this possibility, work from Diano et al. (2006) has demonstrated that peripherally secreted ghrelin is capable of entering the hippocampal formation, where it binds to hippocampal neurons and facilitates synapse formation and long-term potentiation (Diano et al., 2006).

Previous research has demonstrated that meal-entrained rats, who learn to anticipate meals based on circadian cues, show elevated preprandial circulating ghrelin levels. This is in contrast to similarly food-deprived, freely-fed rats who do not show this preprandial rise in ghrelin levels (Drazen et al., 2006). Moreover, data from our lab has shown that vHP ghrelin receptor blockade in meal-entrained rats exhibit a reduction in meal size during the entrainment period, suggesting that the vHP is a site of action for the integration of circulating ghrelin with learned feeding behaviors (Hsu et al., 2015). Given these data, it is possible that in addition to circadian cues, peripheral circulating ghrelin might exhibit a preprandial rise in response to not only social food cues, but also in response to other types of learned food-related cues (e.g. environmental food cues), that in turn allow an animal to anticipate future meals. This hypothesis however, requires further examination. Overall, these data provide evidence for a neurobiological mechanism for learned, social-related feeding behaviors whereby ghrelin secretion from the stomach traverses to the central nervous system and engages GHSR-1As within the vHP to facilitate the acquisition and/or consolidation of mnemonic associations between social cues and food intake.

While the results from the L-cysteine experiment suggest that vHP GHSR-1A-mediated STFP enhancement requires ghrelin-GHSR-1A ligand-receptor binding, the current study did not directly examine the relative contribution of GHSR-1A constitutive activity vs. peripheral ghrelin circulation on learned, social feeding behaviors. Future studies should utilize a combination of inverse agonists for GHSR-1As to silence constitutive activity [e.g., [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P (Holst et al., 2003)] as a selective means to reduce peripheral ghrelin secretion to determine the relative impact of these mechanisms on learned feeding behaviors. It is also important to note that while L-cysteine is a potent means of reducing circulating ghrelin levels, the mechanisms through which L-cysteine reduces peripheral ghrelin levels are currently unknown. Moreover, peripheral L-cysteine administration has been shown to influence central serotonin levels (Invernizzi et al., 1989) and has neuroprotective effects (Liu et al., 2017) via yet unknown pathways, and therefore it is possible that STFP deficits were based on nonspecific actions of L-cysteine. Further investigations involving learned feeding behaviors and inhibition of ghrelin producing P/D1 stomach cells or halting acylation of ghrelin into its active form (e.g. via ghrelin-*o*-acetyltransferase inhibitors, see (Barnett et al., 2010; Teuffel et al., 2015; Wellman et al., 2015)) would complement the present L-cysteine data.

To examine the endogenous relevance of the vHP ghrelin system on STFP, we utilized selective, vHP-targeted RNA interference to chronically knockdown vHP gene expression of GHSR-1A. Results reveal that vHP ghrelin signaling is physiologically relevant for learned, socially-driven feeding behaviors. In comparison to control animals, rats with vHP GHSR-1A KD exhibited impairments in learning the STFP task, as reflected by an equal preference for the Demonstrator-paired flavor of chow compared to the novel unpaired flavor. These effects were likely independent of deficits in olfactory processing and habituation learning, as demonstrated by comparable preferences in both control and vHP GHSR-1A KD animals for a flavor of chow that was placed in the bedding for each animal during the conditioning phase. Collectively, we provide evidence for the endogenous relevance of vHP GHSR-1A signaling and ghrelin circulation on feeding behaviors driven by learned social cues.

Despite a robust impact on learned, social feeding behaviors, we observed no differences in body weight or food intake when the control or vHP GHSR-1A KD animals were maintained on either a normal chow diet or a Western diet high in fat and sugar. This is interesting in light of our previous work showing that pharmacological vHP GHSR-1A activation has potent hyperphagic effects, whereas vHP GHSR-1A blockade reduces chow intake in rats exposed to a meal entrainment schedule (Hsu et al., 2015; Kanoski et al., 2013). It is unknown whether caloric intake and/or body weight would be impacted with greater vHP GHSR-1A KD magnitude (~50% KD observed in the current study) or whether compensatory mechanisms arise to occlude potential food intake and/or body weight effects. Indeed, GHSR-1A whole body knockout mice exhibit reduced food intake, body weight, and adiposity when maintained on a high-fat diet compared to wild-type animals (Zigman et al., 2005). More specifically, Zigman et al., 2005 demonstrated that female, but not male, GHSR-1A KO mice exhibited obesity resistant phenotypes (reduced food intake, body weight, and adiposity) when maintained on a standard chow diet, revealing potential sex dimorphism regarding ghrelin mediated feeding behaviors. Recent data has also demonstrated that female rats, but not male rats show decreased food intake, body weight, and food motivated behaviors following acute lateral hypothalamic GHSR-1A blockade (Lopez-Ferreras et al., 2017). Overall future experiments should examine whether vHP GHSR-1A-mediated STFP effects have similar sex dimorphism. Finally, animals with GHSR-1A KO also exhibit impairments in other types of conditioned feeding behaviors (Blum et al., 2009; Davis et al., 2011; Lamont et al., 2014; Walker et al., 2012), while others have demonstrated that ghrelin signaling in the amygdala mediates fear-related memories and circulating ghrelin is increased in response to stress-related stimuli (Meyer et al., 2014). Thus additional experiments should also examine whether vHP GHSR-1A KD might impact different variations of learned feeding behaviors (e.g. feeding driven by environmental or interoceptive contextual cues) and motivated behaviors.

GHSR-1As are expressed throughout the brain (Alvarez-Crespo et al., 2012; King et al., 2011; Mani et al., 2014; Schele et al., 2016; Skibicka et al., 2011, 2013; St-Onge et al., 2015; Zigman et al., 2006) and we acknowledge the possibility that other brain regions besides the vHP can regulate learned, socially-driven feeding behaviors. For example, the lateral hypothalamic area (LHA) not only expresses GHSR-1A, but also influences feeding in response to

environmental food cues (Olszewski et al., 2003; Petrovich et al., 2012). Our recently published data demonstrated that LHA ghrelin signaling does not mediate feeding in response to learned, circadian cues (Hsu et al., 2015), however whether LHA ghrelin signaling mediates STFP requires further investigation. Other neuropeptides and neurobiological systems also regulate socially-transmitted food preferences, including hypothalamic oxytocin signaling (Olszewski et al., 2014) and hippocampal acetylcholine, CREB, and PDE11A signaling (Countryman and Gold, 2007; Gold et al., 2011; Hegde et al., 2016). In addition, GHSR-1As and 5-HT2C receptors are colocalized in cultured hippocampal rat neurons and 5-HT2C receptor blockade attenuates ghrelin-induced hyperphagia (Schellekens et al., 2015). Whether vHP ghrelin signaling interacts with these systems also requires deeper examination.

5. Conclusions

Overall, the results presented here provide strong evidence in support of ghrelin's role in promoting learned aspects of feeding behavior and extend previous data demonstrating that the ventral subregion of the hippocampus is a critical brain substrate for mediating these effects. Our results corroborate and expand on our previously proposed model whereby hippocampal neural processing represents a broad, comprehensive integrator of learned associations between learned episodic experiences, external food cues, and interoceptive cues that inform about energy status (Kanoski and Grill, 2017). The latter is likely communicated to hippocampal neurons via endocrine and neuropeptidergic signals (e.g., ghrelin, insulin, leptin, orexin, glucagon-like peptide-1), many of which promote hippocampal-dependent memory function (see (Fadel et al., 2013; Van Doorn et al., 2017) for reviews). Here we extend our model by highlighting social interactions as an important episodic mnemonic experience that links external food cues (olfactory) and interoceptive state cues (GHSR-1A signaling) to influence subsequent food preference in rats.

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