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The interoceptive cue properties of ghrelin generalize to cues produced by food deprivation

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Abstract

A number of recent studies implicate the gut-brain peptide ghrelin as a putative "hunger signal". Most of these studies, however, rely on either consummatory behavior (in humans or nonhuman animals) or self-report (in humans) to draw conclusions regarding the orexigenic properties of this peptide. The present study employs the deprivation intensity discrimination paradigm to assess the interoceptive sensory properties of ghrelin in rats. In this paradigm, one group of rats was placed in a training context and presented with sucrose pellets when 24 h food deprived, but not when 1 h food deprived (24+ group). A second group was trained using the opposite sucrose-deprivation level contingency (1+ group). Learning in this paradigm was demonstrated by animals approaching the food delivery location more frequently under their rewarded compared to their non-rewarded deprivation condition (prior to actual pellet delivery). After asymptotic performance of this discrimination was achieved, these animals (1 h food deprived) were administered ghrelin or saline, either i.p. (3 or 6 nmol) or i3vt (0.1 or 1 nmol), placed in the training context, and appetitive responses were measured. Testing was conducted in extinction, eliminating confounding effects of food consumption. Results of these tests showed that 6 nmol i.p. ghrelin and 0.1 and 1 nmol i3vt ghrelin all generalized to a state of 24 h food deprivation, indicating that exogenous ghrelin has sensory properties in common with the stimuli produced by 24 h food deprivation. These results support the notion that endogenous ghrelin contributes to an interoceptive hunger cue, and that this may be a mechanism by which ghrelin influences food intake and appetitive behavior.
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1. Introduction

Several recent findings suggest that release of the gut-brain peptide ghrelin may give rise to a physiological appetite stimulating or hunger signal e.g., [16,28,32,33]. Ghrelin expression is upregulated in the stomach during fasting and increased plasma levels of ghrelin predict meal initiation [27]. Importantly, plasma ghrelin levels also quickly decline after meal termination [9]. In addition, exogenous ghrelin has been demonstrated to increase food intake following either systemic or intracerebroventricular administration in both animals and humans [21,32]. Ghrelin also prevents leptin-induced inhibition of feeding, suggesting that ghrelin and leptin interact competitively [25].

The purpose of the present research is to investigate further the hypothesis that ghrelin is involved in the production of an interoceptive hunger signal. Although administration of ghrelin has been shown to increase food intake in humans and other animals, this finding does not provide

clear evidence that ghrelin also produces interoceptive hunger stimuli. In fact, food intake can be influenced by numerous environmental factors, such as the availability and the perceived attractiveness of food, social factors, and temporal (e.g., meal time) or other cues that are associated with eating e.g., [11–13]. Ghrelin could increase food intake by influencing one or more of these factors, independent of any effect on the interoceptive sensory consequences of hunger per se. Furthermore, receptors for ghrelin have been identified in brain dopaminergic areas that are believed to underlie reward e.g., [1]. These findings suggest that ghrelin might augment food intake and appetitive behavior by modulating the hedonic or positive re-inforcing properties of food [24]. Food intake measures typically do not allow one to dissociate changes in the magnitude of these types of effects with changes in interoceptive hunger stimuli.

Obviously, an alternative index of hunger that is unavailable for use with nonhuman species is subjective report. Because humans have the capacity for introspection, they should be able to report or rate the degree to which they experience the sensory consequences of hunger and satiety. When used carefully, subjective ratings have yielded results that reliably predict eating behavior e.g., [12,15]. Moreover, ratings of appetite have been reported to vary directly with concentration of circulating ghrelin [8]. However, such data do not directly address the hypothesis of whether ghrelin gives rise to the sensory consequences of hunger in humans or other animals. For example, such hunger ratings are likely to depend on one's assessment of local environmental conditions (e.g., proximity to meal time, time since last meal). These assessments may also depend on responses evoked by environmental food cues (e.g., changes in the perceived attractiveness or anticipated sensory qualities of food, elevated arousal level), or by other events that are associated with eating history (e.g., the question "Are you hungry?"). Although such environmental factors might have an important impact on the probability or expected consequences of eating, the degree to which this impacts on interoception of any change in physiological state can be questioned e.g., [20].

The present studies employed a different strategy to assess the possibility that ghrelin produces interoceptive hunger stimuli in rats. This strategy was adapted from our earlier studies that trained rats to use interoceptive stimuli arising from different levels of food deprivation to signal an aversive unconditioned stimulus e.g., [5,10]. In the current studies, we trained rats to use cues arising from different levels of food deprivation (24 h deprivation and 1 h deprivation) as discriminative stimuli for food reward. After asymptotic discrimination was achieved, we first evaluated, in separate test phases, the degree to which systemic and central administration of ghrelin elicited discrimination responding like that following 24 h food deprivation. If ghrelin administration produced a pattern of discriminative responding like that produced by 24 h food deprivation, this would indicate that exogenous ghrelin produced interoceptive sensory stimuli like that produced by 24 h food deprivation.

That is, ghrelin would elicit hunger stimuli similar to 24 h without food.

The measure of cue similarity we employed did not involve food intake or any other form of consummatory behavior. Rats were first trained to use cues arising from different levels of food deprivation as discriminative stimuli for the delivery of sucrose pellets. All sessions took place at the same time of day, near the end of dark phase of the light/dark cycle. One group of rats was trained under conditions where cues arising from 24 h of food deprivation predicted the presentation of pellets, and cues produced by 1 h food deprivation level were not followed by the sucrose reward. A second group of rats received the opposite relationship between food deprivation and sucrose. With this design, evidence of discrimination learning takes the form of more conditioned responding (as indexed by interruption of a photobeam located in the recessed food magazine) when the rats are under their rewarded compared to their non-rewarded food deprivation level.

This training procedure is distinct from that typically used in drug discrimination studies. Drug discrimination designs usually require rats to learn that Response A (e.g., press the left manipulandum), but not Response B (e.g., press the right manipulandum), is followed by re-inforcement under one drug condition, whereas Response B, but not Response A, is reinforced under another drug condition. The re-inforcer, typically food, is delivered if the rat meets some criterion level of performance (e.g., 20 consecutive responses (i.e., fixed-ratio 20)) on the correct manipulandum.

Previous attempts to use drug discrimination designs to establish food deprivation intensity cues as discriminative stimuli have often required hundreds of hours of training to achieve even modest performance e.g., [6]. Part of the difficulty may be attributable to fundamental differences in the properties of drug and food deprivation cue manipulations. For example, it is often claimed that food is more rewarding when it is obtained under a high compared to a lower level of food deprivation e.g., [14,26]. Thus, using a drug discrimination procedure to train food deprivation intensity stimuli would produce greater conditioning and performance of the response trained under high compared to low food deprivation see [19,23]. This differential response tendency is eliminated with the present experimental design because rats make only one response (approach the food cup). In addition, virtually all drug discrimination experiments provide multiple choice response opportunities per training session—a practice that allows the reward outcome of the first choice response to predict that response outcome for the remainder of the session. This procedure necessitates the use of only first choice performance to evaluate discriminative control by the drug cue. However, it seems likely that continued training after the first choice could place reward-produced cues and state cues in competition for control of discriminative responding. If "highly-rewarding" food is more salient than food with lower reward value, one might expect that food cues would be better able to compete with deprivation intensity cues under high compared to low food deprivation. In the present

experiments, the possibility of this type of competition was eliminated by ending each training session after a single rewarded or non-rewarded trial.

After asymptotic deprivation discrimination performance was achieved by both groups, the rats were tested under 1 h food deprivation for generalization between cues produced by 24 h food deprivation and cues produced by administration of ghrelin and isotonic saline, in counterbalanced order. In the first test, all rats were given intraperitoneal (i.p.) ghrelin (3 and 6 nmol) and saline. A second test phase compared the effects of intra-third ventricular (i3vt) administration of ghrelin (0.1 and 1.0 nmol). No sucrose pellets were available during testing, allowing the stimulus properties of ghrelin administration to be assessed independently of effects on the taste or post-ingestive consequences of food. If exogenous ghrelin produces interoceptive cues similar to 24 h food deprivation, rats that were trained to expect reward under 24 h deprivation should exhibit more conditioned responding when tested with ghrelin under 1 h food deprivation when tested with saline. In contrast, ghrelin should not evoke more appetitive responding than saline for rats trained to expect reward under 1 h food deprivation.

2. Methods

2.1. Animals

The subjects were 32 naïve, male, Sprague–Dawley, albino rats that weighed between 230 and 290 g upon arrival in the laboratory from Harlan Inc., Indianapolis, IN. The rats were housed individually in stainless steel cages under a reverse 12 h light dark cycle (lights on 05:00) and given access to standard laboratory chow (Laboratory Rodent Diet; Constant Nutrition 5001) and water ad libitum for 2 weeks prior to training. During training the rats were maintained on a feeding schedule that alternated daily between 23 h ad libitum feeding and 24 h food deprivation. All subjects were weighed daily before training and given ad libitum access to water at all times, except during brief experimental sessions. All procedures for the care and treatment of the rats during this experiment were approved by the Purdue Animal Care and Use Committee.

2.2. Apparatus

All training and test sessions were conducted in eight identical conditioning chambers, constructed of aluminum end walls and clear Plexiglas sidewalls. A recessed food magazine was in the center of one end wall of each chamber. A white noise at approximately 60 dB was used during all training and testing sessions to mask extraneous background sounds. A computer-controlled infrared monitoring system was used to record food magazine entries. One infrared photo transmitter and one receiver was located on each side wall of the recessed food magazine.

2.3. Third intracerebroventricular (i3vt) cannulation

All rats were food deprived for at least 12 h prior to surgery. Following intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) administration, rats were positioned in a stereotaxic frame with the skull leveled horizontally between lambda and bregma sutures. Using stereotaxic coordinates 1.5 mm posterior to bregma and 1.5 mm lateral to the midline, a 24 gauge guide cannula with tip beveled at 45° (Plastics One, Roanoke, VA) was lowered 8.7 mm into the third ventricle at a 10° angle from the vertical as described by [29]. Verification of cannula placement was confirmed by a smooth withdrawal of CSF through the internal cannula. The guide cannula was anchored in position with stainless screws and dental acrylic. When rats recovered from the anesthetic, an analgesic dose of buprenorphine (0.03 mg/kg) was administered subcutaneously before rats were returned to the home cage.

2.4. Cannula placement/patency verification

Cannula placement was verified by visual inspection of cerebrospinal fluid (CSF) upon removal of the stylette at the time of testing. Two rats did not have robust CSF flow at this time. To verify cannula placements in these rats, they were injected with 10 μl of 10% methylene blue into the guide cannula and then sacrificed with pentobarbital (100 mg/kg) and perfused intracardially with 10% formalin. The brains were removed, fixed in formalin and sliced at 80 μm to verify visually that cannula tip was placed inside the third ventricle. Histology revealed that cannula tips were placed well within the ventricular space and blue dye was distributed throughout the third ventricle, cerebral aqueduct and fourth ventricle in both rats whose CSF flow was not robust.

2.5. Drugs

Ghrelin was purchased from Phoenix Pharmaceuticals Inc., Mountain View, CA (rat/mouse ghrelin, Cat# 031-31). Lyophilized ghrelin was dissolved in physiological saline, which also served as the control solution. Ghrelin doses (i.p. and i3vt) were determined based on the results of a pilot studies which assessed hyperphagia elicited by ghrelin during the light phase when food consumption is normally low e.g., [21].

2.6. Procedure

2.6.1. Deprivation intensity discrimination training
Prior to the beginning of training, the rats were assigned to two groups of 16 rats each matched on body weight. For both groups, food deprivation levels alternated each day between 1 h food deprived and 24 h food deprived. On 1 h food deprivation days, rats had free access to food for approximately 23 h before food was removed from the home cage of each rat approximately 1 h prior to the beginning of a training session. On 24 h food deprivation days, rats had no access to food for

approximately 24 h prior to the beginning of the training session. Rats in Group 1+ received five sucrose pellets (45 mg sucrose pellets, P.J. Noyes Company Inc., Lancaster, NH) at the conclusion of each training session that took place under 1 h food deprivation, and received no pellets during training sessions that took place under 24 h food deprivation. Group 24+ received the opposite contingency between food deprivation level and pellet delivery. Although training sessions were always held at the same time of day (15:00), the sessions did not occur everyday to prevent the reward from being delivered according to a single-alternating schedule. The schedule was designed so that the number of transitions from 1 to 24 h and from 24 to 1 h food deprivation during training were equated. In addition, the number of 1–1 h and 24–24 h transitions were also equated.

All of the rats were trained and tested in four squads of eight animals, with each rat in a squad trained assigned to a different conditioning chamber. When the rats were trained under their rewarded level of food deprivation they were placed in the conditioning chambers for 4 min before the sucrose pellets were delivered. During sessions in which rats were trained under their non-rewarded deprivation condition, the feeders operated at the end of 4 min but no pellets were delivered. On both rewarded and non-rewarded training sessions, the rats were removed from the conditioning chambers and returned to their home cages approximately 2 min after feeder operation. Initial training consisted of 56 sessions, with 28 training days under 1 h food deprivation and 28 training days under 24 h food deprivation.

Throughout the experiment, the 4 min period that ended with feeder activation was further subdivided in to twenty-four, 10 s intervals. The percent of these intervals during which the photobeam was interrupted was calculated over the last 1, 2, 3, and all 4 min of each session prior to feeder activation. We considered both number of photobeam interruptions and duration of photobeam interruption as alternative indices of appetitive responding. However, we have no way of determining whether fewer but longer photobeam interruptions indicate more or less appetitive behavior than many but shorter duration photobeam interruptions. The results of pilot work indicated that rats given deprivation discrimination training exhibit both types of behavior. The measure we chose is based on the rationale that any beam interruption indicates more appetitive behavior than no beam interruption, regardless of how often or for how long that interruption occurs during a given measurement period.

2.6.2. Ghrelin test (i.p.)

The rats were tested on 2 days under conditions of 1 h food deprivation. The first test day took place one day after the last 24 h food deprived training day. One day under 24 h food deprivation intervened between the two test days. Testing was conducted during extinction, (i.e., the feeder operated, but no sucrose pellets were delivered). Prior to testing, both Groups 1+ and 24+ were subdivided into two additional groups. These four groups were matched on body weight and dis-

crimination performance over the last two sessions of training. The groups differed with respect to dose (3 or 6 nmol) of ghrelin used during testing. Furthermore, order of treatment with saline and ghrelin was counterbalanced within each dose condition. Ghrelin and saline were administered i.p. in a volume of 0.3 ml approximately 1 h prior to being placed in the conditioning chambers. Immediately following the generalization test, rats were returned to the home cage where food intake (accounting for spillage) was measured for 1 h.

2.6.3. Discrimination retraining

When test phase 1 was completed, each rat was implanted with a cannula aimed at the third cerebral ventricle (i3vt). After recovery from surgery, the rats were trained on their original deprivation intensity discrimination problem until both Groups 1+ and 24+ returned to asymptotic levels of performance. Procedures used for discrimination retraining were the same as those described above for initial discrimination training.

2.6.4. Ghrelin test (iv3t)

Appetitive responding was tested as described above for the i.p. ghrelin test except all rats received i3vt infusions rather than i.p. injections of ghrelin and saline. The i3vt doses used were 0.0 (saline vehicle), 0.1, and 1.0 nmol, injected in a volume of 1 μl, approximately 1 h prior to the beginning of testing. Half the rats in each group (1+ and 24+) were tested with the 0.1 nmol dose and the remaining rats were tested with the 1.0 nmol dose. The order of test treatments (i.e., saline first or ghrelin first) was counterbalanced across groups. Immediately following the generalization testing, rats were returned to the home cage where food intake (accounting for spillage) was measured for 1 h.

2.7. Data analysis

The data from training were evaluated statistically using analysis of variance (ANOVA) with Deprivation level (1 and 24 h) and Blocks of training or retraining, and Sessions (2 sessions per block) as within-subjects factors, and Group (1+ and 24+) as a between-subjects factor. ANOVA for the data from testing employed Test treatment (6, 3 nmol ghrelin, and saline for the i.p. test; 0.1, 1.0 nmol and saline for the i3vt test) as a within-subjects variable with Group and Test order (ghrelin first or saline first) as between-subjects factors. Analyses of simple main effects were used to evaluate significant interactions. α-level for all statistical comparisons was set at 0.05.

3. Results

3.1. Initial deprivation intensity discrimination training

Both groups showed sensitivity to the training contingencies. Fig. 1 demonstrates that rats trained to expect re-
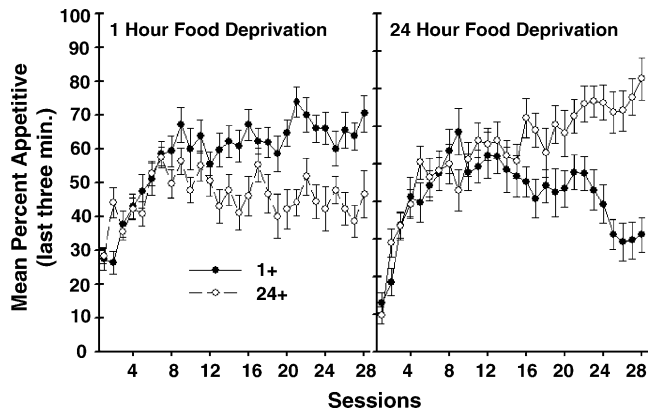
Fig. 1. Mean ± S.E.M. percent magazine entries during the last 3 min of training blocks. The left panel depicts data from 1 h deprivation sessions, while the right panel depicts data from 24 h deprivation sessions.

Fig. 2. Mean ± S.E.M. percent magazine entries during the i.p. generalization tests.

ward when 1 h food deprived (Group 1+) came to exhibit more appetitive behavior during the last 3 min of each session on training days under 1 h food deprivation than did rats that were trained to expect reward under 24 h food deprivation (Group 24+). In contrast, Group 24+ came to respond more than Group 1+ when training sessions occurred under 24 h food deprivation. The data are presented from the last 3 min of each training session because the rats tended to show little appetitive responding during the first minute of each session. The tendency for animals to reduce conditioned responding during time periods that are most temporally distant from the delivery of the un-conditioned stimulus has often been reported with Pavlovian training procedure e.g., [22].

An overall ANOVA obtained significant Group × Deprivation level ($F(1, 26) = 116.96$, $p < .01$) and Group × Deprivation level × Block ($F(13, 338) = 17.89, p < .01$) interactions. Comparison of Group 1+ with 24+ when both groups were trained under 1 h food deprivation yielded a significant main effect of Group ($F(1, 26) = 7.66$, $p < .05$) and significant Group × Block interaction ($F(13, 338) = 3.83$, $p < .01$) confirming that overall of training Group 1+ exhibited more appetitive conditioned responding under 1 h food deprivation than Group 24+, but that the magnitude of this difference varied over blocks. Analysis on each block revealed that Group 1+ responded significantly more than Group 24+ on Blocks 7–9 and Blocks 11–14 (smallest $F(1, 28) = 6.58$, $p < .05$ on Block 7). Comparing both groups on training sessions that were conducted under 24 h food deprivation also yielded a significant main effect of Group ($F(1, 26) = 8.05, p < .01$) and a significant Group × Block interaction ($F(13, 338) = 9.46$, $p < .01$), which confirmed that Group 24+ responded more overall than Group 1+ when training took place under 24 h food deprivation, with the magnitude of this difference also depending on Block. Analysis of performance on each block of training found that under 24 h food deprivation, Group 24+ responding significantly more than Group 1+ on Blocks 10–14 (smallest $F(1, 27) = 5.04$, $p < .05$ on Block 10).
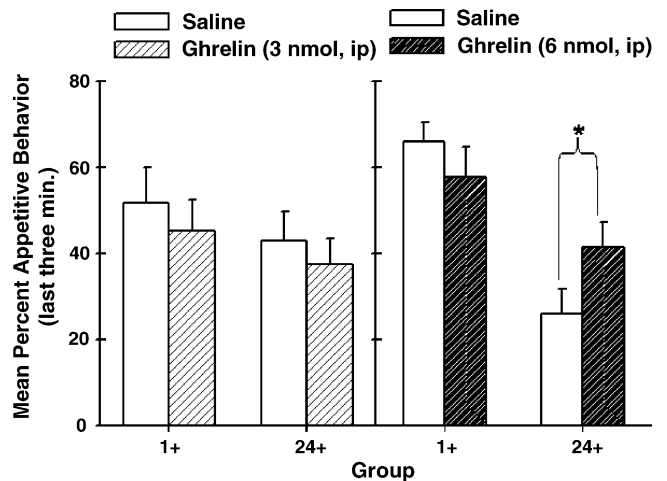
3.2. Ghrelin test (i.p.)

3.2.1. Generalization test

The effects of i.p. injection with 3 and 6 nmol ghrelin on magazine entry performance when both Groups 1+ and 24+ were tested under 1 h food deprivation are shown in Fig. 2. The data depicted were recorded during the last 3 min of each test session. The left panel of Fig. 2 shows that Group 1+ tended to respond slightly more than Group 24+, and that 3 nmol ghrelin, i.p. tended to reduce responding for both groups relative to saline. A somewhat different pattern of results was obtained following 6 nmol ghrelin i.p. Group 1+ continued to exhibit greater overall magazine entries than Group 24+. However, the 6 nmol dose of ghrelin produced more food magazine entries than saline for Group 24+, whereas for Group 1+ treatment with 6 nmol ghrelin i.p. appeared to elicit slightly less responding than did saline.

This pattern of results yielded significant main effects of Group ($F(1, 22) = 5.62$, $p < .05$), Dose, ($F(1, 22) = 5.02$, $p < .05$), and a significant Group × Dose interaction ($F(1, 22) = 5.42$, $p < .05$). ANOVA was also used to evaluate differences for each dose level separately. The main effect of Group was significant with the 6 nmol dose ($F(1, 11) = 5.88$, $p < .05$), but not with the 3 nmol dose ($F(1, 11) < 1$). Thus, when tested under 1 h food deprivation, 6 nmol i.p. ghrelin produced a significantly greater elevation of responding compared to saline for rats that were trained to anticipate sucrose pellets when 24 h food deprived (Group 24+), but not for rats trained to expect those pellets under 1 h food deprivation. This difference was not observed for rats that were administered 3 nmol i.p. ghrelin.

3.2.2. Food intake test

Neither i.p. dose of ghrelin resulted in greater food intake compared to saline for either Group 1+ or Group 24+. ANOVA revealed no significant main effects of Group, Dose,
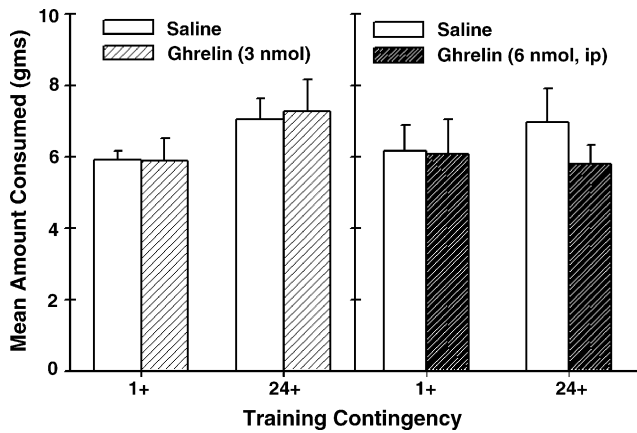
Fig. 3. Mean ± S.E.M. 2 h food intake (g) following i.p. ghrelin generalization testing.

Fig. 4. Mean ± S.E.M. percent magazine entries during the i3vt generalization tests.

or Drug, nor were any interactions involving these factors significant (largest $F(1, 22) = 2.10$, $p > .16$ for Group, all other F's < 1). As depicted in Fig. 3, for both groups substantial eating (>5 g in 1 h) was observed following administration of both saline and ghrelin, respectively. The much higher basal food intake over 1 h in the present experiment is likely due to conditioned eating that resulted as consequence of the long-term exposure to the food deprivation regimen. This conditioned eating may have increased basal food intakes to the point that ghrelin was unable to increase food consumption above those high baselines.

3.3. Discrimination retraining

The rats in Groups 1+ and 24+ responded differentially over the last two blocks of discrimination retraining, which took place immediately prior to testing with i3vt ghrelin. The data reported here, and for subsequent testing with i3vt ghrelin, were collected during the last 2 min of each session, the period where group differences were largest. On the last block that took place under 1 h food deprivation, mean percent magazine entries for Group 1+ was 62.75 and 33.61 for Group 24+. On the last block of retraining under 24 h food deprivation these percentages were 34.56 for Group 1+ and 78.61 for Group 24+. This difference yielded a significant Group × Deprivation level interaction ($F(1, 30) = 53.78$, $p < .01$). Analysis of simple main effects confirmed that Group 1+ responded significantly more than Group 24+ during the last retraining block under 1 h food deprivation ($F(1, 30) = 18.38$, $p < .01$) whereas Group 24+ responded significantly more than Group 1+ over the last block of retraining under 24 h food deprivation ($F(1, 30) = 26.22$, $p < .05$).

3.4. Ghrelin test (i3vt)

3.4.1. Generalization test

Fig. 4 depicts the effects of i3vt ghrelin (0.1 and 1.0 nmol) and saline on mean percent magazine entries during the last
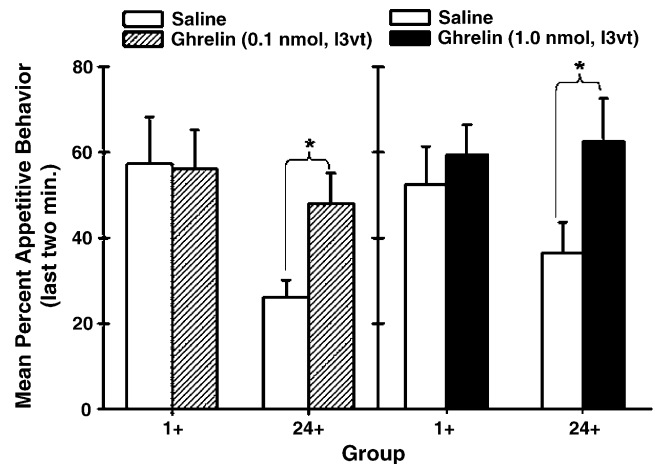
2 min of testing when both Groups 1+ and 24+ were under 1 h food deprivation. As can be seen in Fig. 4, at both the 0.1 nmol and the 1.0 nmol i3vt doses, the effects of ghrelin were quite similar to those of saline for Group 1+. In contrast, magazine entries following ghrelin were substantially higher than following saline for Group 24+. Following saline injection, magazine entries for Group 1+ were much higher than for Group 24+. Differences between Groups 1+ and 24+ were much smaller following either the 0.1 or the 1.0 dose of ghrelin.

These differences yielded significant main effects of Group ($F(1, 23) = 10.58$, $p < .01$) and a significant Group × Drug interaction ($F(1, 23) = 6.99$, $p < .05$). Neither main effect of dose nor any interactions involving dose achieved significance. Analysis of simple main effects collapsed across dose confirmed that Group 24+ exhibited significantly more magazine entries following i3vt ghrelin compared to saline ($F(1, 12) = 12.65$, $p < .01$). For Group 1+ the effect of ghrelin on magazine entry was not significant compared to saline. Furthermore, Group 1+ exhibited significantly more magazine entries than did Group 24+ when both groups were tested following saline injection ($F(1, 23) = 7.97$, $p < .01$), whereas this difference was not significant following injection with ghrelin i3vt.

3.4.2. Food intake test

Fig. 5 demonstrates that neither dose of i3vt ghrelin increased food intake significantly compared to saline for either Group 1+ or Group 24+. ANOVA revealed no significant main effects of Group, Dose, or Drug, nor were any interactions involving these factors significant. As was the case for Experiment 1a, both groups consumed substantial amounts of food following both saline and ghrelin injections. It is likely that this high level of intake reduced sensitivity of the feeding test to the potential augmenting effects of ghrelin on food intake.
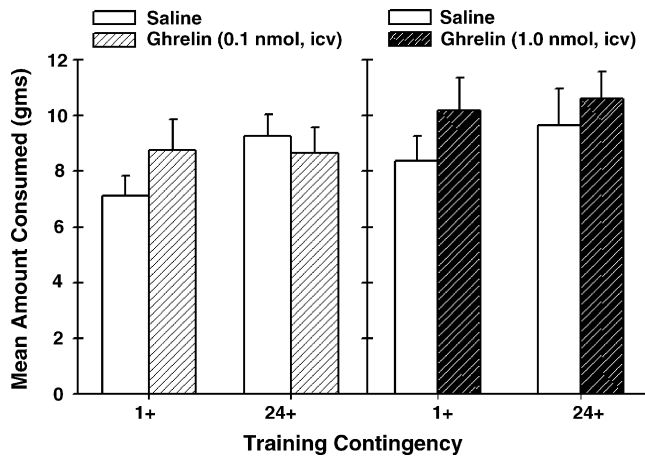
Fig. 5. Mean ± S.E.M. 2 h food intake (g) following i3vt ghrelin generalization testing.

4. Discussion

These findings demonstrate that the effects of exogenous ghrelin on appetitive food magazine approach behavior were dependent on whether the rats had learned to expect the delivery of sucrose pellets when they were 24 h food deprived (Group 24+) or when they were food deprived for 1 h (Group 1+). When conditioned magazine entry behavior was tested under 1 h food deprivation following ghrelin administration, only the rats in Group 24+ showed elevated conditioned responding relative to their saline baseline. This effect was observed after both i.p. and i3vt ghrelin administration. Moreover, the results of Experiments 1a and 1b are not attributable to any potential effect of ghrelin on either the taste of food or on the post-ingestive consequences of eating because no eating occurred during the behavioral testing. Thus, the present results demonstrate that ghrelin can influence appetitive behavior in a manner independent from its potential effects on the rewarding or reinforcing properties of food and eating.

These findings suggest that ghrelin could influence appetitive behavior based on its interoceptive stimulus properties. Our results show that the effects of ghrelin on food magazine approach behavior, relative to those of saline, depended on what the rats had learned previously about stimuli arising from 1 and 24 h food deprivation. During training, the rats in Group 24+ presumably learned that cues arising from 24 h food deprivation predicted the delivery of sucrose pellets, whereas rats in Group 1+ learned this about cues produced by 1 h food deprivation. When the rats in Group 24+ were tested under 1 h food deprivation, treatment with ghrelin apparently re-instated at least some of the cues that were predictive of sucrose pellets during training. Accordingly, treatment with ghrelin evoked more food cup approach responding for the rats in Group 24+ than did treatment with saline. On the other hand, rats in Group 1+ presumably learned little about cues arising from 24 h food deprivation because those cues were not associated with sucrose pellets during training. Thus, for Group 1+, treatment with ghrelin would not necessarily be expected to produce a different level of appetitive responding during testing than would treatment with saline.

The fact that ghrelin did not reduce appetitive responding for Group 1+ and did not elevate responding for Group 24+ to levels like those observed at the end of training after 24 h food deprivation indicates that generalization between exogenous ghrelin and 24 h food deprivation was incomplete. This should not be surprising given that rats were under 1 h food deprivation at the time of testing. Although ghrelin may have elicited some of the interoceptive cues that were experienced by the rats when they were 24 h food deprived, there is little reason to expect that ghrelin would abolish or reduce all of the other conditions (e.g., increased levels of blood glucose, leptin, insulin, CCK, gastric distension, etc.) that may give rise to interoceptive stimuli like 1 h food deprivation. For Group 1+, cues arising from 1 h food deprivation were trained to evoke appetitive responses, whereas cues arising from 24 h food deprivation were not. Therefore, it is not surprising that if ghrelin introduced cues like 24 h food deprivation, these cues would have little ability to reduce response evocation by highly trained, 1 h food deprivation stimuli for the rats in Group 1+. On the other hand for Group 24+, 24 h food deprivation cues, but not 1 h food deprivation cues were trained to evoke appetitive responses. If exogenous ghrelin produced cues like 24 h food deprivation, these cues would have increased responding for Group 24+ against the background of untrained 1 h food deprivation stimuli. Thus, the results are consistent with the hypothesis that ghrelin acts as a hunger signal.

While unexpected, it is also worth noting that ghrelin appears to be able to modulate interoceptive sensory consequences independent of increases in food intake. In the present studies, saline and ghrelin-injected rats consumed similar amounts food, albeit at much higher levels than are typically observed in normal daytime feeding. In fact, we may have inadvertently elicited a form of conditioned eating e.g., [30,31] through repeated 24 h periods of fasting and re-feeding. During the course of training, rats were only presented with food in the home cage after a period of 24 h food deprivation. Presumably, short-term re-bound food intake would have been high on each of these days. Under these conditions one might expect that the stimuli associated with home cage food replacement would become a conditioned stimulus capable of eliciting food intake in the absence of deprivation or physiological need. At the doses we used here, ghrelin was ineffective to increase food intake in the face of conditioned eating. This is not entirely surprising as we have also previously demonstrated that similar conditioned eating can also *block* the anorexic effects an agonist of melanocortin 3/4 receptors, the principal hypothalamic neuropeptide system opposing ghrelin action [4].

An important, but not fully answered, question is the location of the primary receptors that mediate a ghrelin-induced

hunger signal. While ghrelin is secreted from the stomach, ghrelin receptors have been identified in the CNS. Receptors for ghrelin are expressed in the arcuate hypothalamus and co-localized with neurons that express neuropeptide-Y (NPY) and agouti-related protein (AgRP), both of which are known orexigenic peptides e.g., [18,21]. One recent hypothesis is that peripheral ghrelin crosses the blood brain barrier (BBB) and acts on these receptors to facilitate the release of NPY and AgRP e.g., [7,17]. However, this remains somewhat controversial. For example, some investigators have reported that subdiaphragmatic vagotomy blocks peripheral ghrelin-induced hyperphagia in rats [3], whereas others have failed to confirm this observation [2].

Additionally, a central site of action requires a "leaky" BBB or an active transport mechanism. To date, neither of these possibilities has been conclusively demonstrated. While admittedly speculative, it is worth noting that our results are consistent with the hypothesis that peripheral ghrelin acts centrally to increase appetite and food intake. Low doses of ghrelin (0.1, 1.0 nmol) delivered centrally were more effective in the generalization test than was a higher dose (3.0 nmol) delivered peripherally. Finally, some previous reports have also suggested that ghrelin may be produced locally in the CNS e.g., [7]. If this is indeed the case, then ghrelin may act both centrally and peripherally to increase food intake by initiating signals of "hunger." The coordinated mechanism(s) by which these two ghrelin systems may impinge on energy balance regulation remains to be discovered.

Collectively, these findings indicate that both central and peripheral exogenous ghrelin produce interoceptive sensory consequences like those following a period of 24 h food deprivation. This outcome is consistent with the hypothesis that ghrelin is a source of an appetite stimulation or hunger signal.

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