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Ghrelin enhances cue-induced bar pressing for high fat food

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ABSTRACT

Ghrelin is an orexigenic hormone produced by the stomach that acts on growth hormone secretagogue receptors (GHSRs) both peripherally and centrally. The presence of GHSRs in the ventral tegmental area (VTA) suggests that ghrelin signaling at this level may increase the incentive value of palatable foods as well as other natural and artificial rewards. The present investigation sought to determine if ghrelin plays a role in relapse to such foods following a period of abstinence. To achieve this, thirty-six male Long Evans rats were trained to press a lever to obtain a high fat chocolate food reward on a fixed ratio schedule of 1. Following an extinction period during which lever presses were not reinforced, rats were implanted with a cannula connected to a minipump that continuously delivered ghrelin, a GHSR antagonist ([D-Lys-3]-GHRP-6), or saline in the VTA for 14 days. One week later, food reward-associated cues, food reward priming, and an overnight fast were used to induce reinstatement of the lever pressing response. Our results indicate that intra-VTA ghrelin enhances cue-induced reinstatement of responses for palatable food pellets. To the extent that the reinstatement paradigm is considered a valid model of relapse in humans, this suggests that ghrelin signaling facilitates relapse to preferred foods in response to food cues through GHSR signaling in the VTA.

Introduction

Relapse to preferred foods and unhealthy eating habits is a primary concern for people undergoing weight reduction diets (Elfhag and Rossner, 2005; Kramer et al., 1989). This phenomenon is similar to drug relapse in that it can be triggered by the same environmental stimuli: re-exposure to the substance and/or cues associated with it, and exposure to stressful situations (Elfhag and Rossner, 2005; Grilo et al., 1989; Kayman et al., 1990; Gorin et al., 2004). Interestingly, some have demonstrated that cues associated with reinforcing stimuli can elicit feeding responses in satiated animals that similar to those seen when these test animals are hungry (Weingarten, 1983). Experimentally, relapse to food seeking is most frequently studied using an operant conditioning model that is also used in drug addiction research. The paradigm is characterized by 3 successive phases: self-administration training, extinction, and reinstatement (Epstein et al., 2006; Nair et al., 2009a). Over the last decade, the use of this model has facilitated the identification of a number of peptides and neurotransmitters that act centrally to modulate the reinstatement of food seeking, many of which also impact reinstatement of drug seeking (reviewed in Nair et al., 2009a). These include, but are not limited to, orexigenic peptides such as orexin and melanin-concentrating hormone (Cippitelli et al., 2010; Richards et al., 2008; James et al., 2011; Nair et al., 2008, Nair et al., 2009b; Boutrel et al., 2005; Wang et al., 2009). In this context, the role of ghrelin, the only circulating orexigenic hormone currently known, is relatively under-studied.

Ghrelin is a 28 amino acid-long peptide that is produced predominantly in the oxyntic glands of the stomach in times of negative energy balance (reviewed in Castaneda et al., 2010). It is passively transported across the blood brain barrier and acts on growth hormone secretagogue receptors (GHSRs) both inside and outside of the central nervous system (Banks et al., 2002; Diano et al., 2006; Ghigo et al., 2005). Peripheral, intra-cranial and hypothalamic ghrelin administration produces a robust dose-dependent feeding response that is not seen in animals lacking a functional GHSR gene (Lawrence et al., 2002; Wren et al., 2001; Tschop et al., 2000). Ghrelin administration also results in a number of outcomes that are typically observed in the presence of both natural (ex: food) and artificial (ex: drug) rewards, such as increased dopamine (DA) levels in the nucleus accumbens (NAc), increased locomotion and development of a conditioned place preference, suggesting a general role of ghrelin signaling in reward processing (Abizaid et al., 2006; Abizaid, 2009; Jerlhag et al., 2006a/b; Jerlhag, 2008). Likewise, disruption of ghrelin or ghrelin signaling via genetic or pharmacological manipulations can alter the behavioral and physiological response to reinforcers, including operant responding for palatable foods and drugs of abuse (Jerlhag and Engel, 2011; Landgren et al., 2011a; Skibicka et al., 2011a; Skibicka et al., 2011b; King et al., 2011; Landgren et al., 2011b; Clifford et al., 2011; Wellman et al., 2011; Perello et al., 2010; Jerlhag et al., 2010; Jerlhag et al., 2009; Egecioglu et al., 2010; Abizaid et al., 2011). Ghrelin's reward-related effects likely involve the dopaminergic mesolimbic system. Over 60% of DA cells within the ventral tegmental area (VTA) express the GHSR, and ghrelin binding in this area causes

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synaptic re-organization of local DA cells, ultimately increasing the ratio of excitatory (glutaminergic) to inhibitory (gabaergic) afferents to these cells (Abizaid et al., 2006). The resulting reduction in DA neurons' firing threshold and increased accumbal DA turnover might underlie ghrelin's orexigenic effects at this level (Abizaid et al., 2006). Ghrelin's action in the mesolimbic system may be specifically related to adding incentive value to food stimuli (Skibicka et al., 2011a; Egecioglu et al., 2010).

A role for ghrelin in the reinstatement of food seeking is suggested by the fact that ghrelin activates the HPA stress response system, and that stress is intimately linked with food seeking relapse in dieting individuals and rats tested in the reinstatement model (Ghitza et al., 2006; Asakawa et al., 2001; Greeno and Wing, 1994; Grilo et al., 1989; Tassone et al., 2003). Indeed, mice with targeted deletion of the GHSR gene or mice treated with a ghrelin receptor antagonists show lower reinstatement of operant responses to cues previously associated with food (Walker et al., 2012). In addition, caloric restriction, which increases plasma ghrelin levels, facilitates reinstatement of both food and drug seeking (Nair et al., 2009a). Elevated ghrelin levels are also associated with alcohol cravings in people who try to abstain from drinking, and such cravings often precede relapse episodes (Koopmann et al., 2012; Koob and Volkow, 2010). Finally, endogenous ghrelin levels are positively correlated to the reinstatement of cocaine seeking in rats (Tessari et al., 2007).

In the current study, we sought to determine if ghrelin administration within the VTA could restore previously extinguished bar pressing for pellets, a rodent model of reinstatement or relapse in rats. This was examined in response to stimuli that have been associated with the reinstatement of reward seeking behaviors in the past. Specifically, we evaluated the reinstatement of these responses after exposure to a cue previously associated with the reward, the reward itself (i.e. re-exposure to the high fat pellet), or an overnight fast. We chose chocolate-flavored high-fat food pellets as the reward because preferred foods are often sweet and rich in fat and ghrelin preferentially increases intake of sweet and high-fat foods (Shimbara et al., 2004, Disse et al., 2010). The choice of the administration route was based on the presence of GHSRs within this node of the reward system and evidence suggesting the VTA plays a role in the reinstatement of both cocaine and heroin seeking (Abizaid et al., 2006; Guan et al., 1997; Zigman et al., 2006; Wang et al., 2009, Wang et al., 2007; Bossert et al., 2004; Stewart, 1984). In order to limit the effects of endogenous ghrelin levels, which naturally rise in times of negative energy balance at GHSRs located outside of the mesolimbic system, reinstatement was tested in animals that were sated (i.e. not chronically food restricted) and therefore not in negative energy state.

Methods

Subjects & apparatus

Male Long Evans rats (216–375 g, average = 283 g, n = 39) were obtained from Charles River and singled-housed in a room under a reverse dark/light cycle (lights off at 8:00 AM). After a 1-week acclimation period, baseline food intake was measured daily for 5 days. The rats were subsequently food restricted as to maintain 85% of their baseline body weight. All testing was performed during the dark phase, in operant chambers (Coulbourn Instruments®) containing a grid floor, house light, pellet delivery magazine, and two levers 2.5 cm off the floor — one selected as "active" and the other as "inactive". Active and inactive lever presses were recorded by GraphicState software.

Procedure

Autoshaping, training & extinction

Testing began 5 days after the start of food restriction. All animals were subjected to a single 3-hour long autoshaping session during which high-fat chocolate pellets (Bioserv, F05879 — 45 mg, 35% fat) were delivered every 5 min. Each pellet delivery was accompanied by a 2 s light/tone stimulus. Starting on the next day, rats were trained for 3 h every second day, for 24 days (12 training sessions). During training, pressing on the active lever resulted in the delivery of a pellet and exposure to the food cue (light/tone stimulus). Each pellet delivery was followed by a 20 s timeout period, during which pressing on the lever had no consequence. Similarly, pressing on the inactive lever at any point had no programmed consequence. Training sessions were conducted during the morning (9:00 AM–12:00 PM) or afternoon (1:00 PM–4:00 PM), which alternated for each rat. At the start of the first 5 sessions, 3 pellets were placed on the active lever in order to facilitate acquisition. On training days, rats were fed their maintenance food ration (75% of their baseline food intake or about 18–20 g of food) 1 h after the conditioning session. On "off" days, rats were fed 1 h after the start of the dark phase.

One day after the completion of the training phase, rats were exposed to a series of 3-hour extinction sessions, during which lever pressing (active or inactive) had no programmed consequence. Rats received daily extinction sessions until they reached the extinction criterion, defined as pressing on the active lever less than 30 times over 3 h on 3 consecutive days. Rats were then returned to an ad lib feeding schedule until the day of surgery.

Surgery and recovery

Stereotaxic surgery was performed 1 to 6 days after the end of the extinction period. Isoflurane and oxygen were used to anesthetize rats for surgery. A cannula (Plastics One, 3300P-SPC), connected to a minipump (Alzet, model 2002) by a 4 cm long vinyl tube (Plastics One, C312-VT) was then implanted into the VTA. Coordinates for implantation were 5.3 mm posterior to bregma, 2 mm lateral to the midline, and 7.6 mm below the skull surface (Paxinos and Watson, 1986). The cannula was anchored into position by dental acrylic placed around 3 stainless steel screws embedded within the skull. The minipumps, implanted subcutaneously between the shoulder blades, delivered saline, ghrelin (10 nmol; Pi Protemics, PI-G-01), or a ghrelin receptor antagonist, [D-Lys-3]-GHRP-6 (200 nmol; Peptides International, OGH-3656-PI) into the VTA at a rate of 0.5 μL/h for 14 days. Following surgery, rats received a subcutaneous injection of meloxicam (Metacam®, 0.1 mL) to minimize pain and discomfort and were placed in a recovery cage until normal behavioral activity was observed.

Recovering rats were fed mashed food (chow and water mixture) for 1 day following the surgery, and then returned to an ad lib feeding schedule for a period of 4 days, during which food intake was recorded daily.

Reinstatement

Rats were tested for reinstatement of food seeking after a short (two days) and very mild (about 85% of their regular intake) food restriction conditions that were not sufficient to make them lose weight. In fact, rats were about 50 g heavier in average than they were during the training and extinction period of the experiment. Food seeking (i.e. active lever pressing) was reinstated by 1) pre-exposure to the food pellets (food priming), 2) contingent presentations of the light-tone food cue upon lever pressing, or 3) an overnight fast. Rats also received an additional session during which no reinstatement stimulus was used. All rats were exposed to the 4 conditions in a counterbalanced order. In the food priming condition, 5 pellets were delivered in a non-contingent fashion and I second apart and after each rat was placed in the box at the start of the session. In the food cue condition, rats were presented with the tone/light cue 3 times at the start of the session, and upon all active lever presses during the session. In the overnight fast condition, food was removed from the home cage approximately 18 h before the session. Forty-eight hours separated each reinstatement session. With the exception of the tone/light stimulus presentations in the food cue condition, the reinstatement sessions themselves were identical to extinction sessions. Locomotion during these sessions was recorded using an

infrared activity monitor mounted on the ceiling of the cage (Coulbourn Instruments®).

Experimental endpoint and histology

Twenty-four hours following the last reinstatement session, rats were euthanized using sodium pentobarbital (90 mg/mg, i.p.) and perfused with a 0.9% saline solution followed by a 4% paraformaldehyde solution. Brains were extracted and stored in 4% paraformaldehyde for 3 days prior to being transferred to a 30% sucrose solution. Sections containing the cannula tract were then mounted on gelatin-coated slides and stained with cresyl violet to ensure accurate placement of the cannula tip within the VTA. All procedures were conducted in accordance with the Canadian Council of Animal Care (CCAC) guidelines and were approved by the Animal Care Committee at Carleton University.

Results

Statistical analyses

Unless otherwise stated, repeated measures analyses of variance (ANOVA) with time as the within subject factor and drug treatment as the between subject factor were used to analyze food intake, weight change, and the number of operant responses produced at different time points across the experiment. Post hoc Fischer LSD comparisons were conducted when main effects or interaction effects were significant using an $\alpha = 0.05$ as a critical value.

A total of 13 rats were excluded from the study. One rat was excluded for failing to reach the extinction criterion prior to surgery and twelve rats were excluded because of post-surgical complications. Finally, histological examination revealed inaccurate cannula placements in ten rats. While these rats had been assigned to different groups, they did not differ in their behavior from vehicle treated animals. As such we decided to pool them into a group of their own to demonstrate the specificity of VTA infusions in mediating observed behaviors. The final numbers of rats in the saline, ghrelin, and [D-Lys-3]-GHRP-6 and missed cannulae groups were 5, 6, 7, and 8 respectively. Cannula placements are shown in Fig. 1. A cresyl violet stain revealed normal cell morphology around the cannula tract, indicating minimal histological damage. Only data from animals that completed the study were used in the analyses.

Baseline and training measures

Fig. 2 shows the overall operant responses produced animals in the different groups over the training and extinction period. A repeated measures ANOVA determined that, regardless of the group they were assigned to, all animals gradually increased their operant response rate on the active lever during the training period ($F(11,242) = 16.95$, $p < 0.05$, eta square $= 0.70$; Fig. 2A). The acquisition of this task during training, however, was not different between the experimental groups as determined by non-significant main effect for group or interaction effect ($p > 0.05$). Similarly, the number of pellets earned increased significantly over time ($F(11,242) = 15.40$, $p < 0.05$, eta square $= 0.73$; see Fig. 2B), but this increase was not different between groups (non-significant between groups main effect or interaction effect; $p > 0.05$). The number of inactive lever presses did not increase over time ($p > 0.05$; see Fig. 2C).

A gradual decrease in operant responses was seen during the extinction period with all animals achieving the extinction criteria (<10 bar presses during the 3 h testing period) between 8 and 10 days from the start of extinction. There were no significant differences in the number of days to reach extinction criteria between groups as determined by a one way ANOVA ($p > 0.05$; see Fig. 2D).

Repeated measures ANOVAs demonstrated that, regardless of the drug treatment, food intake and body weight gain remained constant over the baseline, training, extinction and postsurgical period. For instance food intake was the same during baseline and in the four days after surgery, and no significant differences were found in the analyses of these data ($p > 0.05$; Fig. 3A). Similarly, while there was a significant

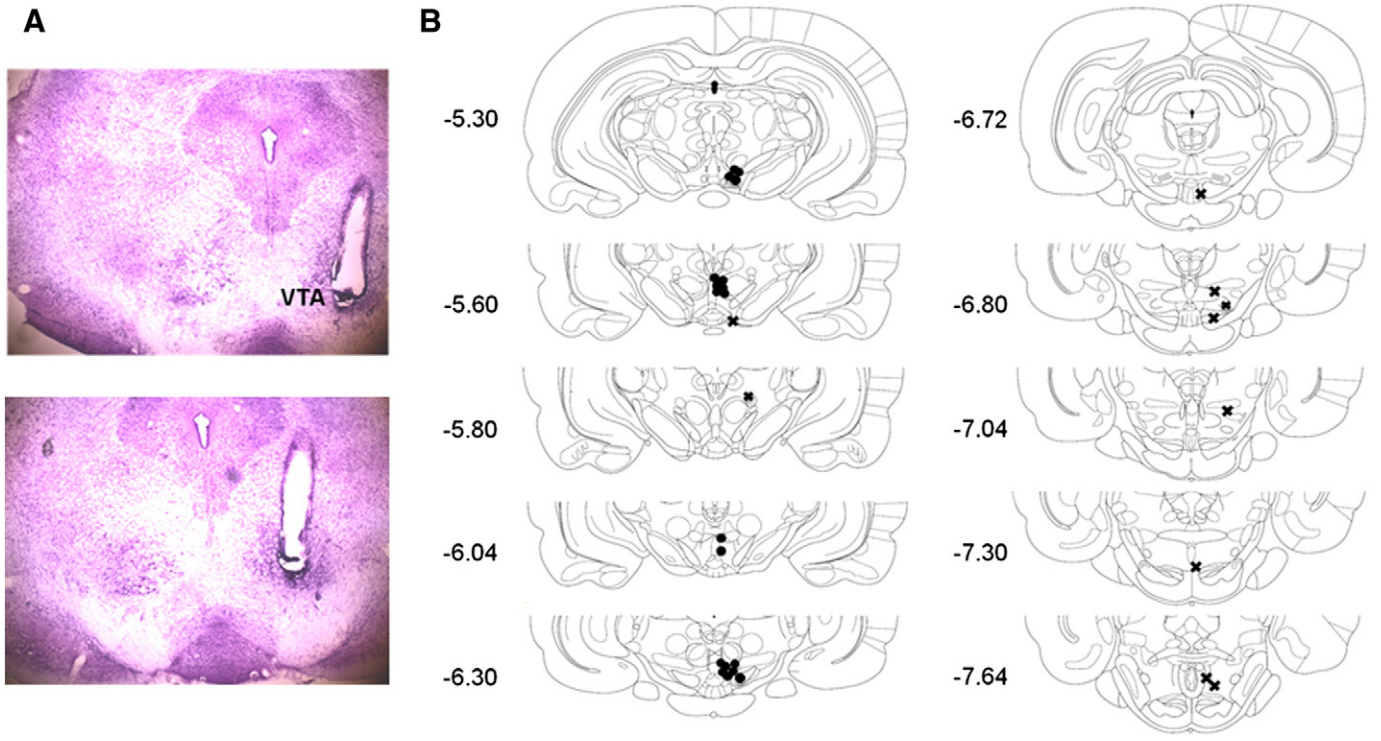

Fig. 1. Accurate (•) and inaccurate (✖) cannula placements for each level on the rostro-caudal axis (Paxinos and Watson, 1986). Panel A shows representative pictures of an accurate (upper panel) and an inaccurate (lower panel) cannula placement. Panel B depicts the placements of animals in this study. As shown in this image, most missed cannula were placed in regions dorsal to the posterior portion of the VTA.

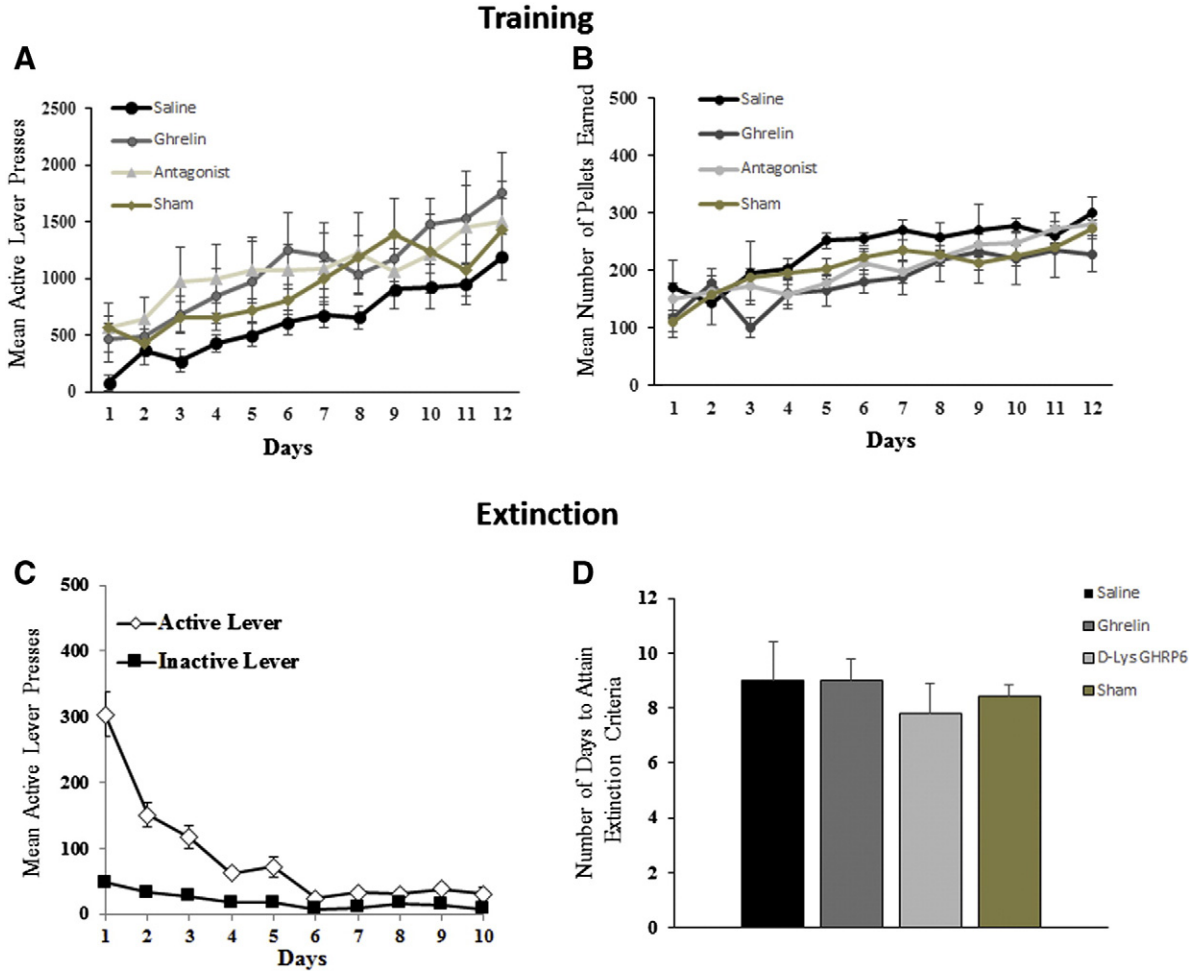

Fig. 2. Acquisition of operant responses for food. Panels A and B depict the mean (+/−SEM) number of active lever presses (Panel A), and pellets earned (Panel B) during training. Panels C shows the overall pattern of responses during extinction in all animals regardless of their group. Finally, Panel D shows the average number of days it took animals in each group to attain extinction criteria. No significant differences were detected between the groups in any of these measures ($p > 0.05$).

increase in the weight of the rats over these phases of the study, the overall weight of the rats in each group did not vary (data not shown, $p > 0.05$). We did detect changes in weight gain during the four days of recovery before reinstatement tests began. A one way ANOVA was used to examine between groups differences in the weight the animals gained during the recovery period, and results from this analysis revealed that [D-Lys-3]-GHRP-6-treated rats gained less weight during this time than saline- and ghrelin-treated rats ($F(3,22) = 3.43$, $p < 0.05$, eta squared = 0.32; LSD post hoc values: $p < 0.05$ in both cases; Fig. 3B).

Reinstatement

The reinstatement of bar pressing responses was evaluated both in terms of total responses, as well as in terms of these responses in comparison with the number of responses each animal produced on the first day of extinction, a phase in which the animals were producing responses while being food restricted.

A repeated measures ANOVA for active lever pressing with reinstatement stimulus as the within subject factor and drug treatment condition as the between subject factor revealed that, overall, rats pressed the lever more when exposed to cues than when exposed to an overnight fast, food priming or no stimuli (significant within groups effect ($F(3,66) = 19.96$, $p < 0.05$, eta squared = .64; see Fig. 4A). A significant interaction effect ($F(9,66) = 2.12$, $p < 0.05$, eta squared = 0.21) revealed a significant effect of drug treatment on active lever pressing in the cue-induced reinstatement condition (see Fig. 4A). Post hoc Fischer LSD tests revealed that under this condition, ghrelin-treated rats had greater responding than [D-Lys-3]-GHRP-6-treated rats ($p < 0.05$). Furthermore, ghrelin treated rats produced more lever presses in response to the cue than rats whose cannulae missed the VTA and saline-treated rats, although this effect did not attain statistical significance ($p = 0.06$) (Fig. 4B). In contrast, the drug treatment did not affect total active lever pressing in the food priming- and fasting-induced reinstatement conditions, and during the session with no reinstatement stimulus, as indexed by comparable responding across treatment groups (food priming, $F(3,22) = 1.08$, $p > 0.05$; fasting, $F(3,22) = .28$, $p > 0.05$; no cue: $F(3,22) = .95$, $p > 0.5$). Inactive lever pressing and locomotor activity did not differ between the treatment groups in any of the reinstatement conditions and during extinction ($p > 0.05$; data not shown).

Finally, in order to determine the effect of the infusions in relation to a testing point in which the animals were being food restricted, we transformed the data in terms of percentage of reinforced operant responses using the last day of training, a time point in which the all of the rats were being food restricted and were bar pressing for food consistently. The rationale behind this was to use these data as a "hungry" baseline to compare the different drug infusions with. As with the analyses of active lever responses, the percent of responses from baseline were highest when animals were presented with the light cue, than when they were primed with food, fasted overnight or placed in the box without a manipulation (significant within groups effect, $F(3,66) = 20.94$, $p < 0.05$, eta squared = .62; see Fig. 5A). Analyses of the percent of hungry baseline responses also revealed a significant interaction effect ($F(9,66) = 2.08$, $p < 0.05$, eta squared = 0.185; see Fig.

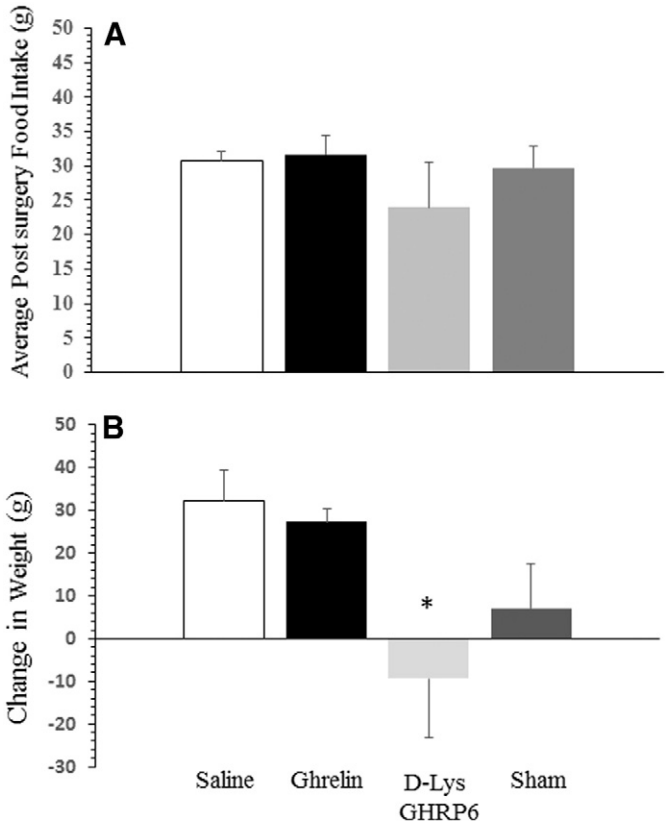

Fig. 3. Mean (+/−SEM) chow intake (Panel A) and weight gain (Panel B) following surgery. As shown in this image, animals in the different treatment groups ate similar amounts of food in the days immediately following surgery, but rats treated with D-Lys GHRP-6 gained less weight than rats in the other groups. (*) denotes $p < 0.05$.

5B). Post hoc Fischer LSD test revealed ghrelin-treated rats had a greater proportion of baseline responses following the presentation of the light cue compared to the saline, [D-Lys-3]-GHRP-6, and sham treated rats ($p < 0.05$). While rats treated with [D-Lys-3]-GHRP-6 tended to have a lower proportion of responses than controls, these did not attain statistical significance ($p > 0.05$; see Fig. 5B).

Discussion

In the current study we evaluated the role of ghrelin receptors in the VTA in mediating cue induced reinstatement of operant responses in satiated rats in comparison with feeding induced by the pellets themselves or by an overnight fast. Our results show that chronic intra-VTA ghrelin administration increased non-rewarded bar pressing responses when in the presence of cues associated with previous availability of high fat pellets. These effects were reflected both in the increased overall number of responses the rats performed in response to the light/tone cue, as well as in in the proportion of responses these animals performed when they were being food restricted. In contrast, rats treated with saline or rats whose cannulae failed to target the VTA did not increase their responses to the light/tone cue. Interestingly rats treated with the GHSR antagonist [D-Lys-3] GHRP-6 seemed to respond less than controls but this effect was not statistically significant.

These data are in line with previous work showing that ghrelin signaling is associated with cue potentiated feeding in mice (Walker et al., 2012). These results are also consistent with those of Tessari et al. (2007), who showed a significant positive correlation between endogenous ghrelin levels and reinstatement of cocaine seeking following exposure to a cocaine-associated cue. Interestingly, our results also support the idea that cues that predict a food reward are more potent elicitors of reinstatement than the food itself, or even an overnight fast. Furthermore, given that there may be different neural substrates underlying cue-, priming-, and stress-induced reinstatement of food seeking (reviewed in Nair et al., 2009a), it is possible that ghrelin selectively targets those that are associated with cue induced feeding. This could account for the fact that ghrelin's effects on cue-induced reinstatement did not generalize to other reinstatement stimuli. Overall, these data support the notion that ghrelin acts on the VTA to enhance the incentive value of environmental cues that are predictive of a food reward and perhaps predictive of other rewards such as drugs or sex.

While ghrelin increased cue induced responding, ghrelin infusions were not sufficient to bring cue induced responding to a level that mimicked the levels of responding seen in animals while they were being food restricted. This is not unexpected given that a food restricted animal would potentially be under the influence of ghrelin acting peripherally as well in several hypothalamic and extra hypothalamic regions simultaneously. Nevertheless, our data do suggest that the VTA is a strong contributor to the effects of ghrelin on feeding responses to cues, and a potential primary target to initiate feeding in response to these cues as suggested indirectly by previous studies (King et al., 2011; Naleid et al., 2005; Skibicka et al., 2011a; Abizaid et al., 2006). It is unlikely that these findings resulted from group differences in hunger or unspecific arousal, since similar effects were not seen following other types of reinstatement stimuli or during an extinction session, and given that this was a design in which animals were tested on all conditions and the order of these conditions was randomized. In addition, drug treatment condition did not affect inactive lever responding or locomotion scores in any of the sessions. Thus, it appears that ghrelin acting on the VTA specifically potentiated the response to cues associated with the high-fat food reward that was formed during training period. It is likely that ghrelin's effects occurred via the activation of local DA cells that project to the nucleus accumbens (NAc). As previously mentioned, ghrelin binding in the VTA lowers the firing threshold of these neurons and increases DA turnover in the NAc (Abizaid et al., 2006). Our results also support the idea that cues that predict a food reward are more potent elicitors of reinstatement than the food itself, or even an overnight fast. Furthermore, given that there may be different neural substrates underlying cue-, priming-, and stress-induced reinstatement of food seeking (reviewed in Nair et al., 2009a), it is possible that ghrelin selectively targets those that are associated with cue induced feeding. This could account for the fact that ghrelin's effects on cue-induced reinstatement did not generalize to other reinstatement stimuli.

Interestingly, an overnight fast failed to elicit reinstatement in any of our groups, including the control group. However, and as with food priming, this stimulus is only effective in reinstating food seeking in metabolically challenged animals, thus making ghrelin or [D-Lys-3]-GHRP-6 effects difficult to detect under the current experimental parameters. It should be noted, however, that in a previous study, [D-Lys-3]-GHRP-6 did not alter fasting-induced reinstatement of heroin seeking (Maric et al., 2012). Importantly, the effects of this manipulation have, to our knowledge, primarily been investigated in drug seeking reinstatement studies (e.g. Maric et al., 2012; Shalev et al., 2001; Shalev et al., 2003). It is therefore possible that food deprivation is simply not an effective reinstatement stimulus for food seeking. A prolonged food restriction paradigm may be more likely to produce a motivational state that allows for an overnight fast to produce reinstatement responses (Carr, 2007). Furthermore, a number of mild stressors known to induce reinstatement of drug seeking in the operant conditioning model do not reliably induce reinstatement of food seeking (Nair et al., 2009a).

The ability of an animal to use environmental cues as a means to finding food involves a myriad of cognitive processes that involve not only the engagement of motivational systems but also circuits associated with memory function and emotional processing. For instance, a hungry rat may be more likely to venture outside of their safe environment to search for food and attend more to stimuli that predict the availability of food, or the presence of a threat. It is therefore not

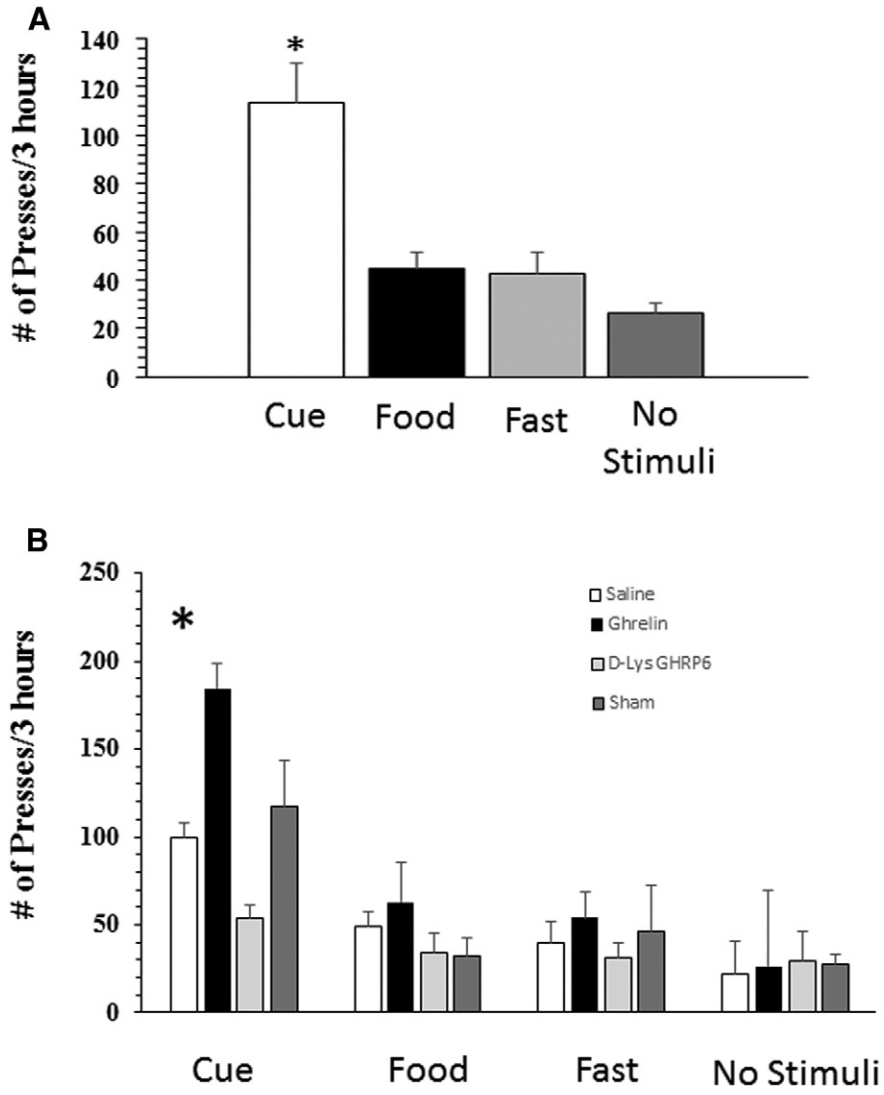

Fig. 4. Reinstatement of food responses. Panel A shows that overall, and regardless of the drug treatment, only the light/sound cues were effective in increasing the number of bar pressing responses during the reinstatement phase of the study. Panel B shows that this increase in bar pressing was primarily seen in rats infused with ghrelin directly into the VTA (* = $p < 0.05$).

surprising that in addition to the VTA, there are other limbic structures that have been implicated in cue induced reinstatement of previously extinguished responses to obtain food. For instance, a number of nuclei within the amygdala, some which contain ghrelin receptors, have been implicated in cue induced feeding (Petrovich and Gallagher, 2003; Mani et al., 2014). Similarly, the ventral hippocampus has been implicated in the formation of associations between environmental stimuli and food availability, and ghrelin infusions into this region also increase cue induced feeding in sated rats (Kanoski et al., 2012). Several studies show that ghrelin facilitates memory acquisition and recall (Davis et al., 2011; Carlini et al., 2004; Diano et al., 2006; Albarran-Zeckler et al., 2012) and promotes dendritic spine formation and long-term potentiation in the hippocampus (Diano et al., 2006; Cahill et al., 2014).

It should be noted that chronic treatment with [D-Lys-3]-GHRP-6 did not significantly decrease reinstatement of active lever pressing. While the average number of responses was lower in rats treated with this compound, and there was a tendency for rats treated with this GHSR antagonist to respond less in proportion to the hungry baseline, these were not statistically different from saline or from animals with cannulas in regions adjacent to the VTA. Several studies report changes in food intake following VTA GHSR activation or antagonism (Skibicka et al., 2011a; Abizaid et al., 2006; King et al., 2011). Discrepancies between our findings and those of others may be explained by differences in the administration protocol. Most studies examined the effects of acute ghrelin or GHSR antagonist infusions, and observed effects lasting from 1 to 3 h (Skibicka et al., 2011a; Abizaid et al., 2006). Only one study examined the effects of chronic intra-VTA ghrelin/[D-Lys-3]-GHRP-6 treatment (King et al., 2011). The changes in food intake observed in that study took place over the entire infusion period, which lasted 14 days. Thus, it is conceivable that the window, during which food intake was measured in the current study (4 days within the first week following surgery), did not allow for the detection of acute (immediately after surgery) or chronic effects of VTA GHSR manipulations on feeding. Given that [D-Lys-3]-GHRP-6-treated rats in our study gained the least amount of weight after the surgery, one cannot argue that the drug was not working in these animals. One could argue, however, that decreased weight gain in these animals put them in a metabolic state that would elicit a higher rate of responses than would normally would be expected, and one that would not be different from control animals. Alternatively, one could argue that given the generally low rate of responses that are seen in this paradigm under normal conditions, make it difficult to detect an effect that would lower an already low rate of lever presses. This is more likely as others have shown that [D-Lys-3]-GHRP-6 can decrease progressive ratio responding for palatable pellets in rats (King et al., 2011).

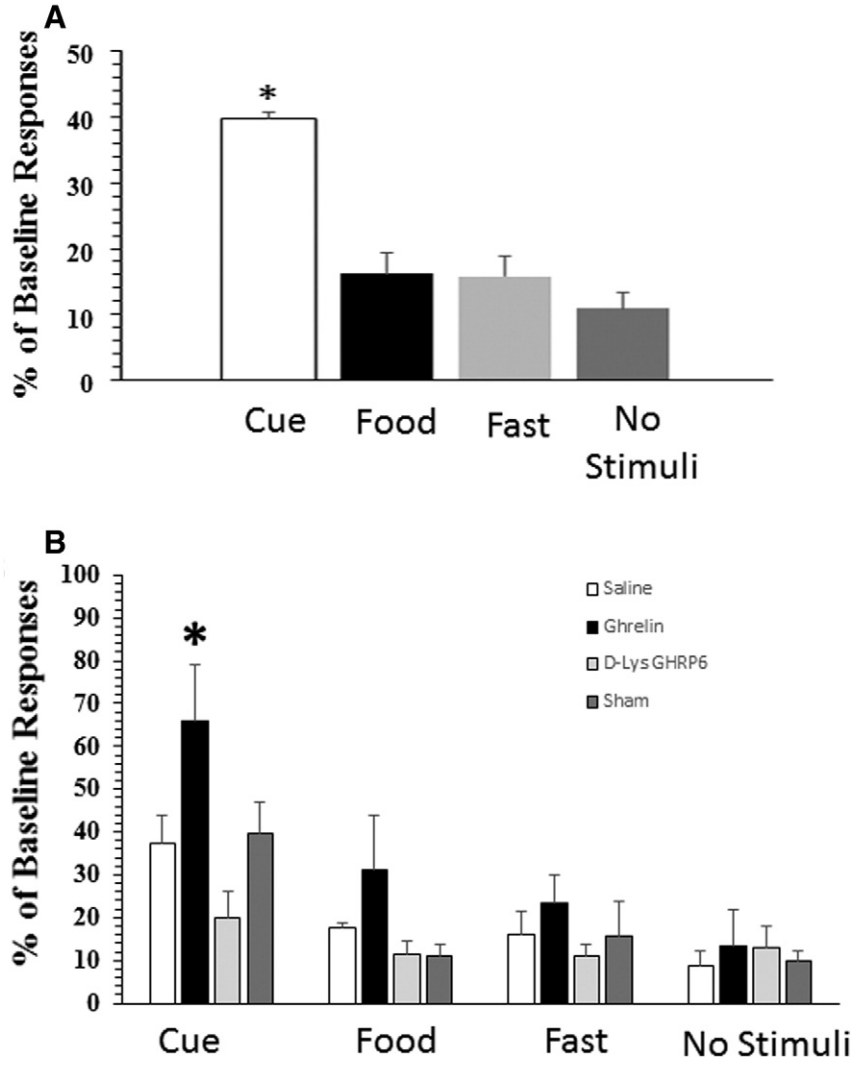

Fig. 5. Reinstatement of food responses in relation to a "hungry" baseline. Panel A depicts the Mean (+/− SEM) the overall percentage of responses in relation to the number of responses seen during the baseline, when animals were being food restricted and therefore metabolically challenged by food restriction. Panel B depicts the proportion of these responses from those reinforced responses obtained on the last training session by animals in the different treatment groups, (* = $p < 0.05$).

One potential caveat of the current study is the possibility that the relatively large volume of the solutions infused may have caused leakage into surrounding tissues. However, previous studies have shown that ghrelin's acute orexigenic effects in the VTA are not present in rats when the cannula is placed in areas adjacent to the VTA (Abizaid et al., 2006). This indicates limited effects of ghrelin in areas surrounding the VTA and any effect of ghrelin or D-Lys-3-[GHRP-6] following minipump infusion is therefore most likely attributable to their effects directly in the VTA. Our study concurs with the notion that the effects seen in the current study are specific to the VTA given that rats whose cannulae were implanted outside of this region did not show differences from saline treated rats in their reinstatement responses to cues.

In conclusion, our findings indicate that ghrelin facilitates the reinstatement of food seeking in response to cues, and that this effect can be generated by ghrelin acting on GHSR in the VTA. To the extent that the reinstatement paradigm is considered a valid model of relapse in humans (Epstein et al., 2006; Nair et al., 2009a), our data would suggest that ghrelin acting on the VTA is an important element in the mechanisms that increase food cravings, and even cravings to addictive drugs in the face of cues associated with them. Our data suggest that drugs blocking ghrelin or its receptors may be useful in decreasing the incentive value of cues associated with high calorie foods, and prevent over consumption of these foods.

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