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Central ghrelin receptor stimulation modulates sex motivation in male rats in a site dependent manner

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ABSTRACT

Ghrelin, a hormone produced primarily by the stomach, has been associated with motivational processes that include reward-seeking behaviors. In male laboratory mice, elevation of ghrelin levels enhances some aspects of sexual motivation and behavior, whereas in other experiments with male mice, rats, and other species, ghrelin treatment or food deprivation decreases sexual motivation and/or behavior. The present tested the hypothesis that stimulation of ghrelin receptors in different brain regions have opposite effects on male sexual motivation and behavior. To do this we examined appetitive and consummatory sex behaviors of male rats with a truncated ghrelin receptor (FHH-GHSR$^{m1/Mcwi}$), and that of their WT (FHH) littermates. We also examined the effects of ghrelin or the ghrelin antagonist D-Lys-GHRP6 delivered into the VTA or the MPOA on appetitive and consummatory sex behaviors in male Long Evans rats. Results demonstrate that rats with a truncated ghrelin receptor, or rats that are food deprived, show deficits in anticipatory sex. Furthermore, although ghrelin does not further stimulate sex anticipation in rats when infused into the VTA, intra-VTA infusions of D-Lys-GHRP6 into the VTA further decreases in sex anticipation in food deprived rats. In contrast, ghrelin delivery into the mPOA decreased sex anticipation compared to saline or D-Lys-GHRP6 infused rats. Overall, these data suggest that ghrelin receptor signalling is important for full expression of appetitive sex behaviors. Within the VTA, ghrelin may act to enhance sex motivation, while acting on the mPOA to decrease sex motivation and promote foraging.

1. Introduction

The 28 amino-acid peptide hormone ghrelin is produced mainly in the gastric mucosa of the stomach and circulates throughout the body to regulate food intake, body weight, energy expenditure, and glucose homeostasis (Kojima et al. 1999; Tschop et al. 2000). In laboratory rodents, plasma ghrelin concentrations fluctuate across the light/dark cycle, with ghrelin concentrations peaking at around the onset of the dark phase, when animals eat most of their food (Drazen et al. 2006). Peaks in ghrelin concentrations, however, can be entrained to the time of the day in which meals are available, and are associated with food anticipatory activity (Blum et al. 2009; Cummings et al. 2001; LeSauter et al. 2009). Once feeding begins, ghrelin concentrations drop rapidly, a process associated with satiety (Cummings et al. 2001). Ghrelin's binds to its only known receptor, the growth hormone secretagogue receptor-(GHSR) to generate its biological actions. The GHSR is expressed in a number of tissues including the ovaries, pancreas, stomach, thyroid, and testes (Guan et al. 1997; Howard et al. 1996; Papotti et al. 2000). In addition, the GHSR is expressed in the central nervous system, including in several hypothalamic nuclei associated with the regulation of food intake and energy balance (Guan et al. 1997; Zigman et al. 2006). Ghrelin acts at these sites to stimulate the release of orexigenic peptides such as Neuropeptide Y (NPY) and agouti related peptide (AGRP), and to promote food intake and adiposity (Nakazato et al. 2001; Tschop et al. 2000).

In addition to hypothalamic nuclei, receptors for ghrelin are detected in a number of regions within midbrain and limbic areas that are associated with learning, emotion and motivation processes (Zigman et al. 2006). One of these regions, the ventral tegmental area (VTA), contains relatively abundant expression of GHSRs, that is primarily localized on dopamine cells (Abizaid 2009; Abizaid et al. 2006). Ghrelin administration into the VTA increases action potential frequency, and results in increased dopaminergic release and turnover in the nucleus accumbens (NA) (Abizaid et al. 2006; Jerlhag et al. 2006). Furthermore,

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ghrelin activity within this pathway increases both food intake and food seeking behaviors in response to cues that predict food rewards (Abizaid et al. 2006; Jerlhag et al. 2010; King et al. 2011; Naleid et al. 2005; St-Onge et al. 2016). Moreover, ghrelin receptor antagonists delivered into the VTA attenuate peripheral ghrelin-induced food intake and the intake of food after an overnight fast (Abizaid et al. 2006). Ghrelin also enhances the hedonic value of drugs including alcohol, nicotine, cocaine, amphetamines, and opiates (Abizaid et al. 2011; Davis et al. 2007; Dickson et al. 2011; Tessari et al. 2007; Wellman et al. 2005). Together these data support the notion that ghrelin may promote a state of increased motivation that generalizes to a variety of reinforcers, including sex.

Recent evidence supports a role for ghrelin in sexual motivation. Male mice, given either peripheral injections of ghrelin or infusions of ghrelin into the VTA, showed an increased preference for receptive female mice when given the choice between an estrous female and a male (Egecioglu et al. 2016; Prieto-Garcia et al. 2015). The same treatment also enhanced aspects of consummatory sexual behavior, including a reduction in the latency to mount a receptive female, as well as an increase in the number of and duration of mounts. In addition, mice with targeted deletion of the GHSR (GHSR KO mice) or wild type (WT) mice treated with a GHSR-1a antagonist either peripherally, into the VTA, or into the laterodorsal tegmentum (LTDT) display decreased preference for receptive female mice, as well as decreased mounting and intromitting, behaviors. These behaviors were restored with L-DOPA treatment (Egecioglu et al. 2016; Prieto-Garcia et al. 2015).

In contrast to those data, it is well established that most animals will forgo reproduction when faced with increased energetic demands, or when food is scarce (Schneider 2004; Schneider et al. 2013). In males rats this is reflected by an increased latency to approach receptive females in animals that are food deprived (Caquineau et al. 2012). This effect may be mediated in part by ghrelin given that there is emerging substantial evidence of ghrelin modulating the hypothalamic-pitiuitary-gonadal (HPG) axis inmale and female rodents (Bertoldi et al. 2011; Farkas et al. 2013; Fernandez-Fernandez et al. 2005; Kluge et al. 2009). Furthermore, intraperitoneal injections of ghrelin inhibit mating vocalizations in male mice in response to receptive females and had no effect on the preference for the odour of female bedding (Shah and Nyby 2010). Moreover, sexually naïve rats infused with ghrelin into the third ventricle displayed significantly increased latencies to mounting, intromission, and ejaculation when allowed to interact with a sexually receptive female, and these effects were prevented with ICV treatment with a GHSR receptor antagonist (Babaei-Balderlou and Khazali 2016). Although these discrepancies may in part be due to differences in species or in the operational definition of sexual motivation, they do bring into question the role of ghrelin in modulating male sexual behavior.

To explain these paradoxical effects, we propose that there are independent neural substrates underlying the motivational and consummatory components of sexual behavior (Beach 1976; Everitt 1990), and that ghrelin acts on these in parallel as a means to managing the trade-off between feeding and reproductive function as reviewed recently (Schneider and Deviche 2017). Using this model, one could argue that ghrelin acts on the VTA to stimulate a general state of motivation, while targeting regions associated with reproductive function to inhibit them and bias behavior towards foraging. In this sense, while food deprivation may have an overall effect inhibiting sex behavior to promote feeding, ghrelin infused directly into the VTA should have an overall stimulating effect on sexual motivation and behavior, and antagonists into the VTA may further decrease sex motivation in food deprived animals. In contrast, increased ghrelin signalling in brain regions associated with sexual behaviors may result in decreased sexual motivation and copulatory behaviors as reported in previous studies (Babaei-Balderlou and Khazali 2016; Caquineau et al. 2012; Jarmon and Gerall 1961; Shah and Nyby 2010). One potential target for this suppressive effect is the medial preoptic area (mPOA), a region of the brain important for the integration of sensory and hormonal information, and critical for the elicitation of sexual motivation and consummatory behaviors in males of a number of avian and mammalian species (Will et al. 2014). Importantly, this region contains ghrelin receptors (Zigman et al. 2006), and is a region where other peptide metabolic signals like neuropeptide Y inhibit sex behaviors in male rats (Kalra et al. 1988).

To address these issues, we conducted a series of experiments that examined the role of ghrelin receptor function on sexual behavior. We first evaluated sexual behavior and sexual motivation in fawn hooded hypertensive rats with a point mutation that results in a truncated GHSR protein (FHH-GHSR$^{m1/Mcwi}$), as well as that of their wild type (FHH-WT) littermates. These rats show some minor deficits in some metabolic parameters, reduced responses to stimulants and intracranial self-stimulation (Clifford et al. 2012), as well as a decrease in palatable food intake in a manner similar to GHSR KO mice (MacKay et al. 2016), and as such represent a good model for the study of ghrelin signalling and male sexual behavior. As part of this experiment, we observed the behavior of these rats towards sexually receptive females when they were sexually naïve, and across several subsequent tests to determine whether mutations to the ghrelin receptor also results in alterations in appetitive and consummatory sexual behaviors in sexually experienced animals. In the second experiment, we compared differences in sexual behaviors in sexually experienced rats with free access to food that received ghrelin directly into the VTA with that of rats that had been food deprived and that received a GHSR antagonist delivered onto this region. Finally, we examined the effects of ghrelin receptor stimulation in the mPOA on sexual motivation.

2. Methods

2.1. Experimental subjects

Male Long Evans rats purchased from Charles River farms (St. Constant, Que) and male rats from the FHH-GHSR$^{m1/Mcwi}$ strain and their FHH WT littermates served as subjects in these experiments. The FHH-GHSR$^{m1/Mcwi}$ rats originated from breeding pairs purchased from Transposagen (Lexington, KY) and bred at Carleton University with FHH WT rats obtained from Charles River Farms. In addition, Long Evans female rats weighing 250–270 g were purchased from Charles River Farms (St- Constant, QC) to serve as stimuli animals. Rats weighed between 350 and 450 g at the onset of the study and were housed individually under standard laboratory conditions on a reverse 12 h light dark cycle with lights on at 7:00 PM. Rats had free access to food and tap water, and were exposed to environmental enrichment (i.e. Plexiglas tubes, pumpkin seeds, chew blocks etc). One week after arrival, female rats were bilaterally ovariectomized (OVX) through lumbar incisions under a mixture of 3 parts isoflurane to 1 part oxygen (3:1). Females were treated post-operatively with subcutaneous injections of 3 cm^3 physiological saline, and 1 mg/kg Metacam. These stimulus females were primed with estradiol benzoate (EB; 10 μg/0.1 mL sesame oil) and progesterone (P; 500 μg/0.1 mL sesame oil) administered 48 and 4 h prior to sexual behavior tests respectively. All steroid compounds were obtained from Steraloids (Newport, RI). EB (10 μg) and P (500 μg) and dissolved in 0.1 mL sesame oil under low heat for approximately 30 min, and stored at room temperature. All procedures were approved by the Carleton University Animal Care Committee and followed the guidelines of the Canadian Council for Animal Care.

2.2. Anticipatory and consummatory sex behavior in males

All sexual behavior training and testing occurred in bi-level chambers (Mendelson and Pfaus 1989), during the middle third of the dark cycle. These chambers were designed to facilitate the experimenter's view of the full behavioral repertoire of sexual behaviors (Mendelson

and Pfaus 1989; Pfaus et al. 1999). On the test day, experimental male rats were placed in the bottom compartment of the bi-level chamber for a10 minute habituation period. Next, hormone-primed females were introduced on the top of the bilevel chamber and interactions observed for 30-min. This procedure was conducted for a total of five training sessions with receptive females, each training session occurring 4 days apart. All tests were video-recorded with a Sony Handycam, and subsequently scored using the Behavioral Observation Program customized for rodent sexual behavior (Cabilio, S., unpublished computerized event recorder). Anticipatory behaviors were defined as the number of level changes and rears (active seeking behaviors) during the 10-min period before the receptive female was placed in the bi-level chamber (Mendelson and Gorzalka 1987). Consummatory behaviors measured were measured as: frequency of mounts, intromissions, and ejaculations, as well as their latency (Pfaus 1999a, 1999b).

2.3. Experiment 1

In this study, we evaluated differences in sex behavior between rats with a truncated ghrelin receptor and their WTR littermates. Male FHH-GHSR$^{m1/Mcwi}$ rats (n = 6) and male WT littermates (n = 6) were used for this experiment. Of these, one WT and two FHH-GHSR$^{m1/Mcwi}$ rats had to be removed from the experiment because they were exposed to a female that was not receptive at a training trial and therefore were not able to show a full display of consummatory behaviors during the session and appetitive behaviors in subsequent sessions. Data from the first session were analyzed using independent group t-tests to compare the performance of naïve FHH-GHSR$^{m1/Mcwi}$ rats and WT littermates. Changes in behavior across sessions was then compared between WT and FHH-GHSR$^{m1/Mcwi}$ using repeated measures ANOVAs with genotype as the between groups variable, and training session as the within groups variable. The critical value for significance was set at $\alpha = 0.05$.

2.4. Experiment 2

Ghrelin targets the VTA to increase sexual behaviors in sexually naïve mice (Prieto-Garcia et al. 2015). In contrast, food deprivation, a manipulation that is associated with elevated levels of ghrelin, appears to attenuate male sexual behavior in rodents (Jarmon and Gerall 1961; Kalra et al. 1988; Schneider et al. 2013). In this experiment, we sought to examine the effects of intra-VTA infusion of ghrelin on rats that had free access to food, or the effects of the ghrelin receptor antagonist D-Lys-GHRP6 on sexual motivation and sexual behavior in sexually experienced fasted male rats. To do this, male Long Evans rats (n = 24) purchased from Charles River Farms and housed under standard laboratory conditions, were exposed to repeated tests of sexual behavior in bi-level chambers as described above. After the fourth training session, rats were anaesthetized with isofluorane (5%) mixed with oxygen and, following aseptic protocols, had a small hole made on to the skull and a guide cannulae aimed 1 mm above the VTA was implanted using coordinates obtained from the Paxinos and Watson rat brain atlas to target the VTA unilaterally (Paxinos and Watson 1998). The coordinates used were: Anterior-posterior (AP;-6 mm); Lateral (L; 2 mm), and Dorso-ventral (DV; 7 mm), with the cannula being inserted at a 5° angle. The tip of the cannulae was aimed at the central part of the VTA so as to target both sides of this structure. In the past, this method has been effective in producing feeding and food seeking behaviors in animals (Abizaid et al. 2006; St-Onge et al. 2016). The cannulae were secured to the skull with stainless steel screws and dental acrylic cement. After the surgery, animals were treated with metacam (2 mg/kg) once per day for three days to minimize pain and discomfort produced by the surgical procedure. Of the 24 rats operated, two died following surgical complications, and the data from six others were not included in the analyses given incorrect cannulae placements. Data from 17 rats were therefore used for the final statistical analyses.

After a one week of recovery, rats were tested on the bi-level cages again. A group of these rats were fasted overnight before being tested (n = 8) and the remainder were tested under ad lib conditions (n = 9). Thirty minutes before being tested, food deprived rats were infused with either D-Lys-GHRP6 (Peptides International, KY; 10 μg/1 μl, over a period of 3 min) or saline into the VTA. Rats in the ad lib fed condition were infused either with saline or human acylated ghrelin (Tocris, St. Louis; 1 μg/1 μl, over a period of 3 min). All drugs were infused via an indwelling cannula that extended 1 mm beyond the guide cannulae. The dose of ghrelin that we chose for this experiment was based on its effectiveness in producing food intake responses in rats (Naleid et al. 2005). The dose of D-Lys-GHRP6 was used because of its effectiveness in reducing food intake when infused icv (Asakawa et al. 2003). Following the infusions, rats were placed in the bilevel chambers and behavior was recorded as in Experiment 1. One week later, all rats were re-tested using a counterbalanced design, such that food deprived rats that received saline in the first post-operative test, received D-Lys-GHRP6, and vice versa. Similarly, animals that received ghrelin on the first postoperative test received saline on the second test and vice versa. Differences in all measures were then analyzed using repeated measures ANOVAs with drug being the repeated measures variable. The critical value for significance was set at $\alpha = 0.05$.

2.5. Experiment 3

Food deprivation and icv administration of ghrelin result in a decrease in sexual behaviors in sexually naïve animals (Babaei-Balderlou and Khazali 2016; Jarmon and Gerall 1961; Kalra et al. 1988). Here we proposed that a potential target for these effects is the medial preoptic area (mPOA), a region that is important for the expression of male sexual behavior, and one where metabolic hormones like NPY can act to inhibit these behaviors (Kalra et al. 1988; Will et al. 2014). In this experiment, we evaluated the effects of ghrelin delivery into the mPOA on male sexual behavior, hypothesizing that ghrelin, like NPY, would be a signal of negative energy state and as such, one that would act on the mPOA to decrease the expression of sexual behaviors in sexually experienced rats. To test this hypothesis, Long Evans rats (N = 24) purchased from Charles River farms (St-Constant, QC) and trained as in Experiments 1 and 2 were used. Of these, five rats had incorrect cannula placements and their data was therefore removed from the statistical analyses, leaving an N = 19. As in Experiment 2, surgical implantation of cannulae occurred following the fourth testing session. In this surgery, rats had bilateral 22-gauge stainless steel guide cannula implanted 1 mm above the mPOA, using the following coordinates from bregma on a flat skull: AP −0.6, L ± 0.5, DV −7.0 mm. Both blocking and infusion cannulae were 28-gauge, and were 1 mm longer than the guide cannulae. After recovery, rats received bilateral infusions of either saline, ghrelin (1 μg/1 μl) or D-Lys-GHRP6 (10 μg/1 μl) into the mPOA and placed in the bi-level chamber for their last session with receptive females, as described on Experiments 2. All animals in this experiment received all drug treatments in a counterbalanced design, with treatments conducted one week apart. As such, differences in all measures were then analyzed using repeated measures ANOVAs with drug treatment as the within subjects group variable. The critical value for significance was set at $\alpha = 0.05$.

3. Results

3.1. Experiment 1.- rats with truncated ghrelin receptors display reduced anticipation to predictable sexual encounters with receptive females

Fig. 1 shows the latency to approach a receptive female on the first session, when all rats were sexually naïve (see Fig. 1B). In addition, this figure depicts anticipatory behaviors in these rats across training sessions (see Fig. 1C). Results showed that, there were few if any rears or level changes observed in male rats from either strain on the first trial in the bi-level chambers. Once receptive females were placed in the

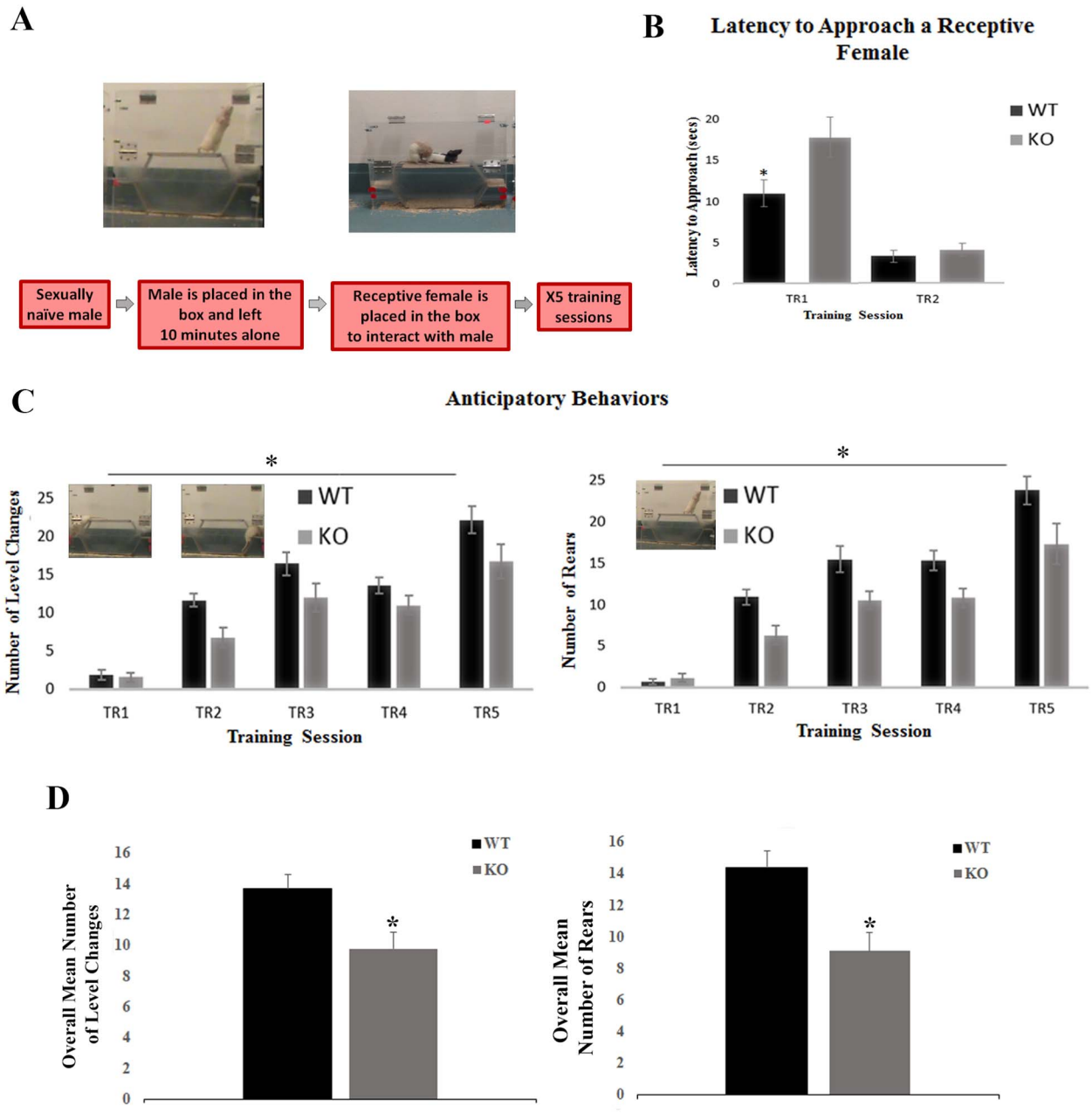

Fig. 1. Role of GHSR in sexual motivation. Fig. 1A shows a schematic of the experimental paradigm, where FHH-GHSR$^{m1/Mcwi}$ (n = 4) and FHH-WT (n = 5) rats were used to study anticipatory and consummatory sex behaviors. As shown in Fig. 2B, sexually naïve FHH-GHSR$^{m1/Mcwi}$ showed a longer latency to approach a novel receptive female than sexually naïve FHH-WT rats, an effect that disappeared on the second encounter ($p < 0.05$). After five training sessions, FHH-GHSR$^{m1/Mcwi}$ showed lower anticipation to sexually receptive females compared to WT rats as reflected in a lower number of level changes and rears in the 10 min preceding the presentation of the receptive female on the last day of testing (Fig. 1C and D). * = significant, $p < 0.05$.

chambers, however, we observed that FHH-GHSR$^{m1/Mcwi}$ took longer to approach the females than FHH WT rats ($t(7) = 2.98$, $d = 1.99$ $p < 0.05$). This difference, however, was only evident on the first trial. The latency to approach females in this task decreased dramatically on Test 2 and no strain difference was observed (see Fig. 1B).

Analysis of data across testing session revealed that the frequency of rearing in the 10 min prior to presentation of the receptive female was increased by the second test and, with the exception of test 4, increased across tests in both genotypes (Significant main effect of test, $F(4, 14) = 79.029$, $p < 0.05$, $\eta p^2 = 0.85$). Although FHH-GHSR$^{m1/Mcwi}$ rats showed increased rearing across tests, overall they showed a lower number of rears than FHH WT rats (Significant genotype main effect, $F(1, 14) = 7.44$, $p < 0.05$, $\eta p^2 = 0.35$; see Fig. 1, panels C and D). There was no significant interaction effect ($p > 0.05$). The same pattern of results was obtained when analyzing level changes in anticipation to a receptive female in these rats. Indeed, all rats increased level changes in anticipation of receptive females over the tests (Significant within groups main effect, $F(4, 14) = 78.988$, $p < 0.05$, $\eta p^2 = 0.84$), but FHH-GHSR$^{m1/Mcwi}$ consistently showed significantly lower number of level changes than their WT littermates (Significant genotype main effect, $F(1, 14) = 7.71$, $p < 0.05$, $\eta p^2 = 0.35$; see Fig. 1, panels C and D).

In contrast to anticipatory behavior, few differences in consummatory sex behaviors were oserved (See Fig. 2). FHH-GHSR$^{m1/Mcwi}$ rats showed the same latency to ejaculate and the same number of total ejaculations during the first testing session, as the FHH WT rats, but required a significantly higher number of intromissions to achieve ejaculation than FHH WT rats ($t(7) = 2.49$, $p < 0.05$; see Fig. 2a and

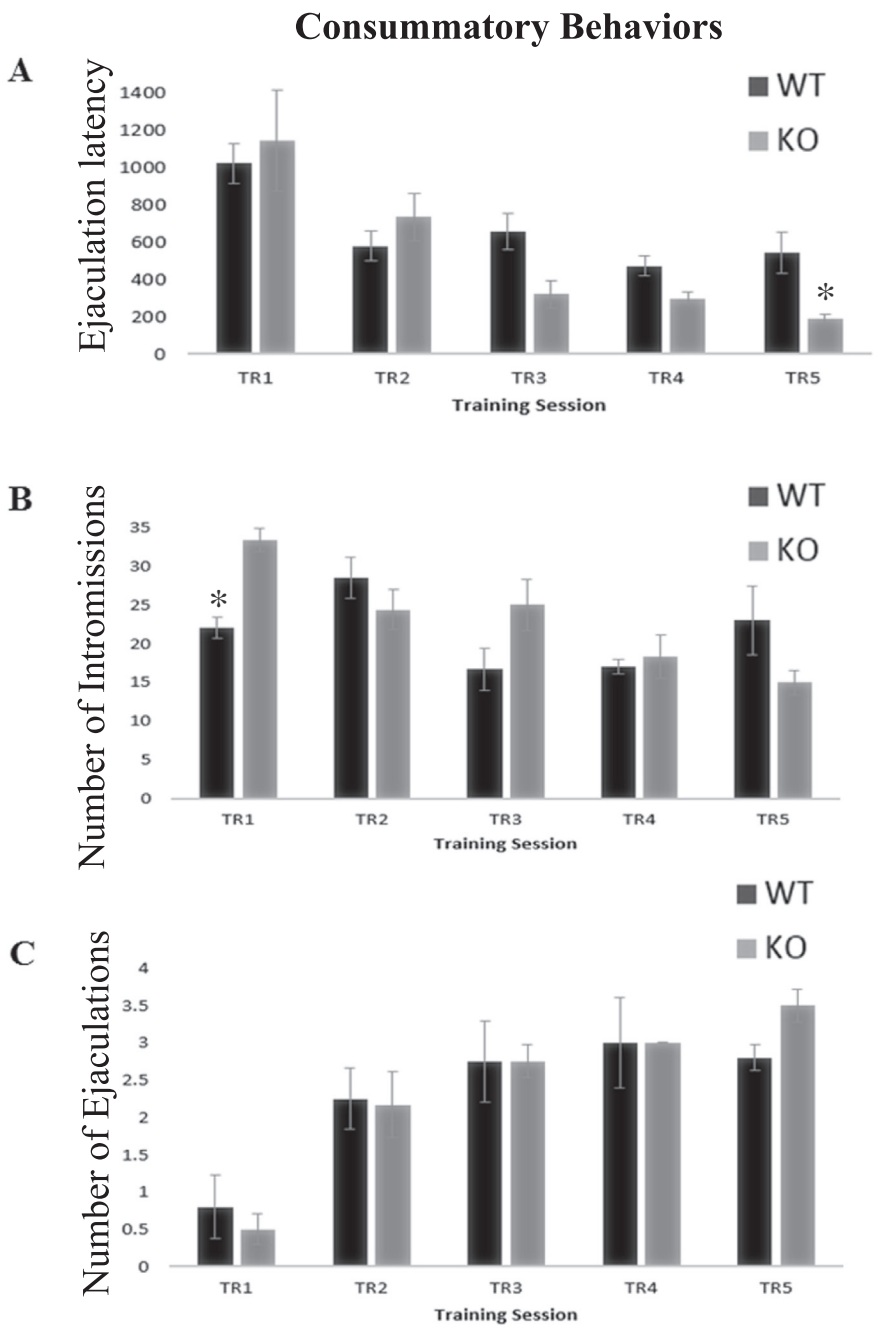

Fig. 2. Consummatory sex behaviors of FHH-GHSR$^{m1/Mcwi}$ (n = 4) and FHH-WT (n = 5) rats throughout the testing trials. As shown in figs. 2A,B and C, FHH-GHSR$^{m1/Mcwi}$ did not differ much from FHH-WT rats in most measures recorded. FHH-GHSR$^{m1/Mcwi}$ did showed less intromissions during the first testing trial ($p < 0.05$), but this effect was not evident in the subsequent testing trials. * = significant, $p < 0.05$.

b). This difference was not detected after the first session. By contrast, there were no differences in the latency to ejaculate between FHH-GHSR$^{m1/Mcwi}$ and FHH WT rats on the first testing trial, but FHH-GHSR$^{m1/Mcwi}$ rats did display a decreased latency to ejaculate that was statistically significant by the 5th testing trial. This decreased latency was observed in spite of FHH-GHSR$^{m1/Mcwi}$ rats having the same number of mounts, intromissions and ejaculations as FHH WT rats during the testing trial ($t(7) = 2.48$, $d = 1.36$, $p < 0.05$; Fig. 2A).

3.2. Experiment 2.- blocking ghrelin receptors in the VTA attenuates level changes in anticipation of a predictable sexual encounter in food deprived animals

As in Experiment 1, all rats showed a dramatic increase in anticipatory behaviors across the training phase and a robust display of level changing and rearing behaviors as well as consummatory behaviors in the last session prior to surgery, (data not shown). Fig. 3 shows cannulae placements, anticipatory and consummatory sexual behaviors in ad lib fed rats that received ghrelin or saline, as well as rats that were food deprived and received D-Lys-GHRP6 or saline. As seen in Fig. 3B, food deprivation decreased the overall number of level changes regardless of drug treatment (significant diet regime effect, $F(1,16) = 24.9$, $p < 0.05$, $\eta p^2 = 0.61$). Further analyses (Post hoc Fisher LSD) demonstrated that ghrelin delivery did not further increase the number of level changes in adlib fed animals ($p > 0.05$), but the ghrelin antagonist D-Lys-GHRP-6 did enhance the reduction in level changes seen in fasted rats (significant within groups effect, $F(1,7) = 7.7$, $p < 0.05$, $\eta p^2 = 0.52$; see Fig. 3B). Rearing behavior was not influenced by either food deprivation or by ghrelin or D-Lys-GHRP6 infusion ($p > 0.05$). Similarly, consummatory behavior was not affected by either food deprivation or drug treatment ($p > 0.05$; see Fig. 3C).

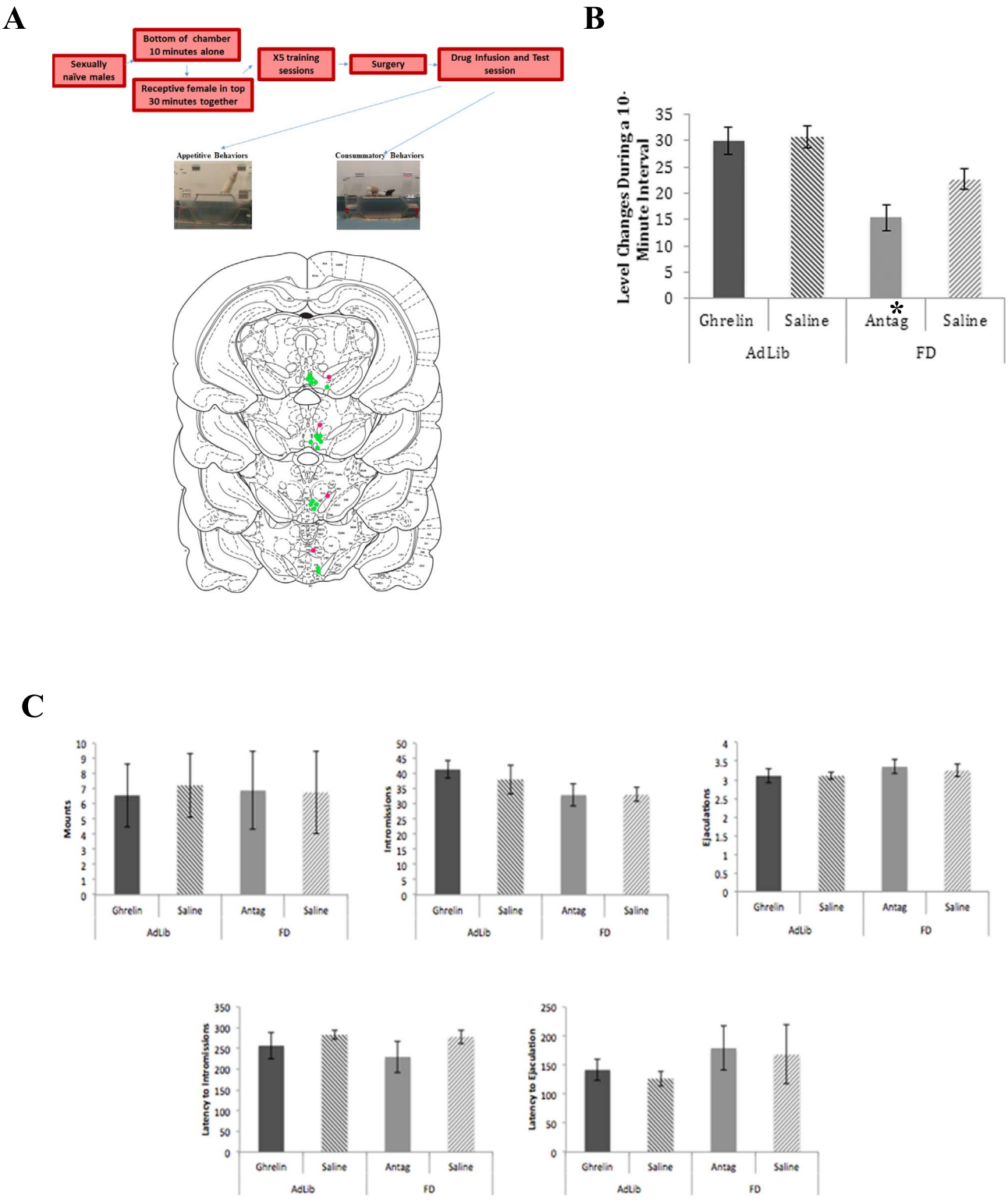

Fig. 3. Effects of unilateral intra-VTA ghrelin (1 μg/1 μl) on sex behavior in ad lib rats and intra-VTA infusions of the GHSR receptor antagonist D-Lys-GHRP6 (10 μg/1 μl) on sex behavior in fasted rats. Fig. 3A shows cannula placements with green dots pointing to cannula placed in the VTA, and red dots pointing to cannula that ended outside of the VTA. The total number of animals used in this study was $n = 17$. As shown in Fig. 3B, the 24 h fast produced a marked decrease in sex motivation as measured by anticipatory level changes. Ghrelin infusions were not effective in enhancing anticipatory level changes, but the GHSR antagonist D-Lys-GHRP6 further decreased the number of anticipatory level changes in fasted rats. As shown in Fig. 3C, neither ghrelin nor D-Lys-GHRP6 influenced consummatory sex behaviors. * = significant, $p < 0.05$.

3.3. Experiment 3.- ghrelin infused into the MPOA inhibits locomotor activity in anticipation of a receptive female

As in Experiment 2, all animals showed robust anticipatory and consumatory behaviors prior to cannula implantation. Fig. 4 shows cannulae placements, anticipatory and consummatory sex behaviors in animals given saline, ghrelin, or D-Lys-GHRP6 infusions into the MPOA. As seen in Fig. 4B, the number of level changes in anticipation of a

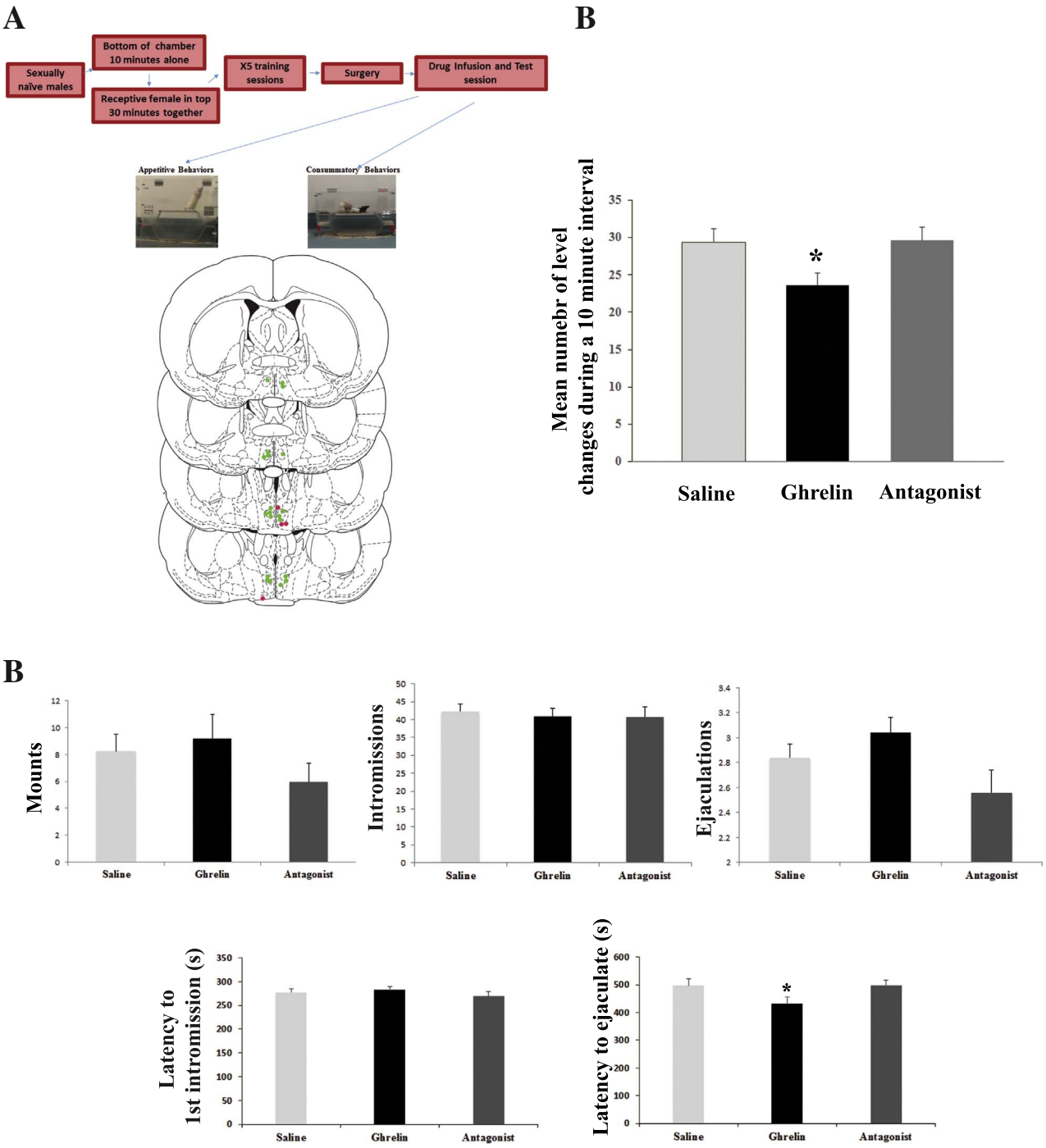

Fig. 4. Effects of bilateral intra-mPOA infusions of ghrelin (1 μg/1 μl) or the GHSR receptor antagonist D-Lys-GHRP6 (10 μg/1 μl) on sex behavior in male rats. Fig. 3A shows cannula placements with green dots pointing to cannula placed in the mPOA, and red dots pointing to cannula that ended outside of the mPOA. Cannula placements are only depicting unilateral placements. The total number of animals used in this study was $n = 19$. As shown in Fig. 4B, ghrelin infusions into the mPOA decreased anticipatory level changes on the test day compared to saline and GHSR antagonist treated male rats. As shown in Fig. 4C, intra-mPOA ghrelin infusions shortened ejaculation latency but not the number of mounts intromissions or ejaculations compared to saline or D-Lys-GHRP6 infusions. * = significant, $p < 0.05$.

receptive female was significantly lower when rats were treated with ghrelin than when they were treated with saline or the ghrelin receptor antagonist D-Lys-GHRP6 (significant within groups effect, $F(1,18) = 7.87$, $p < 0.05$, $\eta p^2 = 0.304$). In contrast, anticipatory rears were not influenced by the drug treatment ($p > 0.05$). Infusions of ghrelin or D-Lys-GHRP6 did not affect any measures of consummatory behaviors collected in this study ($p > 0.05$) with the exception of ejaculation latency, where ghrelin treated rats had a shorter ejaculation latency than saline or antagonist treated rats ($p < 0.05$; see Fig. 4C).

4. Discussion

Results from the present experiment support the idea that GHSR is important for the initiation of sexual behavior in sexually naïve male rats. These data also support the contention that the GHSR plays a significant role in the full expression of male sexual motivation, even in animals that are sexually experienced. Moreover, these effects are site specific such that GHSR signalling in the VTA may serve to enhance sexual motivation whereas GHSR signalling in the mPOA decrease

sexual motivation as measured by level changes in anticipation of exposure to a receptive female.

Results from the first experiment, in which male rats bearing a mutant form of the GHSR were more hesitant to approach a receptive female on their first exposure, suggest that GHSR signalling is important for the initiation of social interaction between the sexually naïve males and novel females. These data are consistent with findings showing that GHSR KO mice had a longer latency to approach and investigate receptive females on initial exposure, as did mice treated with a GHSR antagonist whereas male mice treated with ghrelin showed a shorter latency to approach a receptive female (Egecioglu et al. 2016). In contrast to the deficits in copulatory behaviors observed in male GHSR KO mice, we observed few differences in copulatory behavior between FHH-GHSR$^{m1/Mcwi}$ and FHH WT male rats, in the current study. Indeed, the only substantial difference that was observed was an increase in the number of intromissions, which was associated with neither a decrease in the number of ejaculations, nor in the latency to ejaculate, suggesting that sexually naïve male FHH-GHSR$^{m1/Mcwi}$ require and produce a faster rate of intromissions in order to ejaculate.

A role for GHSR signalling in sexual motivation was also evident in sexual experienced rats in the current study. Although both FHH-GHSR$^{m1/Mcwi}$ and their WT littermates showed the expected increase in anticipatory behaviors during the 10 min before female presentation across tests, FHH-GHSR$^{m1/Mcwi}$ rats, made fewer rears and level changes. These results are interpreted as lower anticipation of sex, and are similar to deficits in food anticipation observed in this strain of rats (MacKay et al. 2016), in GHSR KO mice, (Blum et al. 2009; LeSauter et al. 2009), and in deficits in cue-induced feeding in rats treated with GHSR antagonists (St-Onge et al. 2016). In contrast, consummatory behaviors were, for the most part, similar in rats from both strains after the first exposure to sexually receptive females. Thus, following the first trial, both FHH-GHSR$^{m1/Mcwi}$ and FHH WT rats displayed the same number of intromissions and ejaculations across the ensuing testing trials. FHH-GHSR$^{m1/Mcwi}$ rats did show a lower latency to ejaculate compared to FHH WT rats, but did not increase the total number of ejaculations during the 30 min testing trials. It can therefore be concluded that sexually experienced rats with a truncated GHSR show motivational deficits in response to factors that predict the presence of a sexually receptive partner, but the mutation to the GHSR does not significantly impair sexual performance once the sexually receptive partner is present.

The mechanisms underlying the deficits in sex motivation observed in sexually experienced FHH-GHSR$^{m1/Mcwi}$ male rats possibly reflect deficits in the activity of the mesolimbic dopaminergic system. Indeed, the GHSR is present in dopamine cells within the VTA, and stimulation of these receptors results in increased dopamine cell activity, and increased release and utilization of dopamine in the nucleus accumbens (NA) (Abizaid et al. 2006; Jerlhag et al. 2007; Quarta et al. 2009). Ghrelin infusions into the VTA increase feeding, and in particular the consumption of palatable foods, whereas infusions of GHSR antagonists into this region prevent peripheral ghrelin induced feeding, decrease the intake of preferred foods and attenuate cue induced feeding (Abizaid et al. 2006; King et al. 2011; Perello et al. 2010; Skibicka et al. 2011; St-Onge et al. 2016). Motivational deficits have also been observed in rats with this same point mutation, including deficits in food anticipatory activity, reward based feeding, sensitization to cocaine, and decreased intracranial self-stimulation (Bayerl et al. 2016; Clifford et al. 2012; MacKay et al. 2016; Wei et al. 2015; Wellman et al. 2012).

The results of Experiment 2 support a role for GHSR signalling in the VTA in sex motivation. Although intra-VTA infusions of ghrelin did not increase anticipatory level changes or rears preceding the presence of receptive females in response in ad lib fed rats, we did observe a decrease in these behaviors in rats that were food deprived and received infusions of the GHSR receptor antagonist D-Lys-GHRP6. These data only partially support work by Prieto-Garcia et al. who previously showed that intra-VTA infusions of ghrelin enhanced sexual behavior in sexually naïve mice (Prieto-Garcia et al. 2015). However, in the current study the effect of intra-VTA ghrelin administration was assessed in sexually experienced rats showing high, perhaps ceiling, levels of these behaviors. In contrast, food deprivation decreased anticipatory behaviors, and this effect was enhanced by intra-VTA infusions of a GHSR antagonist.

The results from the VTA study clearly show that, while ghrelin may act on the VTA to enhance sex anticipation, food deprivation decreases overall sex anticipation, an effect enhanced by direct application of a ghrelin antagonist. Given that ghrelin concentrations increase following a fast, these results may seem paradoxical. One potential resolution to this situation is to suppose that under negative energy balance states, ghrelin acts on the VTA to enhance the overall motivated state of an animal, while at the same time acting in other brain regions to inhibit sex motivation and produce behaviors more important for survival, such as finding food. This would explain why the GHSR antagonist infused into the VTA further decreased sex motivation in food deprived rats. One route through which ghrelin could have this effect is by acting in hypothalamic regions that control energy balance, like the arcuate nucleus (ARC), where ghrelin could stimulate the release of NPY into regions of the brain that control sex behavior and function such as the mPOA (Kalra et al. 1988). Alternatively, ghrelin could directly target regions that are important for the onset of male sexual behavior in rodents including the olfactory bulbs, the medial preoptic area, and ventromedial hypothalamus where GHSR expression has been reported (Zigman et al. 2006). The mPOA is of particular interest given that it integrates hormonal, olfactory and metabolic information to regulate sex behaviors in male and female rodents (Coria-Avila et al. 2014; Dominguez 2009; Numan and Stolzenberg 2009; Will et al. 2014). In the current study, we found that when ghrelin was infused into the MPOA it inhibited sex anticipation in sexually experienced male rats. One could therefore propose that under negative energy balance, ghrelin may have an overall suppressive effect on sex behavior in spite of stimulating an overall motivated state through its actions on the VTA. This may be mediated by direct actions of ghrelin in the hypothalamus where it may bias motivation towards food by inhibiting the action of the mPOA on sex motivation.

Ghrelin infusions in the mPOA also decreased the latency to the first ejaculation. It is possible that ghrelin influences ejaculation latencies through an interaction with serotonin, which is associated with states of satiety and sexual inhibition (Coria-Avila et al. 2014; Dominguez 2009; Fernandez-Guasti et al. 1992; Gorzalka et al. 1998; Gorzalka et al. 1990; Numan and Stolzenberg 2009; Will et al. 2014). The mPOA receives serotonergic projections from the midbrain raphe nucleus, and infusions of serotonin directly into the mPOA or NAcc of male rats substantially prolong ejaculation latencies. Conversely, decreasing serotonin levels with a 5-HT1A autoreceptor agonist decreases the latency for ejaculation (Ahlenius et al. 1981; Fernandez-Guasti et al. 1992). Ghrelin may act presynaptically to decrease serotonin levels in the mPOA and hence shorten ejaculation latencies. We could even argue that the effects of intra-mPOA ghrelin on shortening ejaculation latencies reflect an increase in mating efficiency that would allocate more time to feeding or food seeking behavior.

Changes in adiposity are coupled tightly to reproductive function, and fat stores are particularly important in regulating physiological and behavioral reproductive responses. One could argue that rats lacking functional GHSR would be more likely to show alterations in reproductive behaviors given that they are smaller and leaner than WT rats. This question is particularly pertinent to Experiment 1, where, given the effects of the mutation (FHH-GHSR$^{m1/Mcwi}$ are slightly leaner than WT FHH rats)), reduced sex motivation in GHSR KO may be the result of responses to differences in the concentrations of other metabolic hormones such as leptin. It is important to note that ghrelin not only increases food intake, but also facilitates the utilization of carbohydrates over fat. Moreover, deficits in ghrelin signalling can severely compromise adaptations to nutritional challenges like a 24 h fast (Blum

et al. 2009; Zhao et al. 2010). Thus, one could also argue that impaired ghrelin signalling results in lower glucose levels in FHH-GHSR$^{m1/Mcwi}$ rats that are food deprived, and that this leads to decreased sex motivation. The central mechanisms recruited to deal with rapid drops in nutritional signals involve the hypothalamic melanocortin system as well as the brain stem regions that integrate information from ascending vagal afferents and that from circulating signals like glucose and fatty acids (Grill and Hayes 2009; Grill and Kaplan 2001; Ritter et al. 2011). These regions include the area postrema and the nucleus of the solitary tract (NTS)(Grill and Hayes 2009). Experiments probing for the relative contribution of ghrelin signalling in these regions to the regulation of reproductive behavior in male and female mammals are required for a better understanding of the neural effects of ghrelin on reproductive function and behavior.

The contribution of ghrelin signalling in the VTA to sex anticipation, however, is unveiled in experiment 2 where all groups of rats were weight-matched before the experiment. In this experiment, the deprivation resulted in weight loss and likely in a drop in the concentrations of leptin and other signals that also influence reproduction. A reduction in the concentration of these signals may be the key factors producing the overall reduction in sex anticipation that is further decreased by ghrelin receptor antagonism. Thus, GHSR signalling in the VTA (but not in other regions like the hypothalamus or brain stem) may help sustain sex motivation.

FHH-GHSR$^{m1/Mcwi}$ male rats displayed deficits in sex anticipatory activity throughout all testing sessions, but these rats still displayed increasing levels of anticipatory activity. This suggests that ghrelin signalling is important for the full display of sex anticipatory behaviors, but not critical for the display of sex motivation. A similar set of results has been presented in studies examining the role ghrelin receptor signalling in food anticipation using mice and rats with mutations to the gene encoding the GHSR (Blum et al. 2009; LeSauter et al. 2009; MacKay et al. 2016). Nevertheless, it is important to note that anticipatory processes are generated by the combined action of mesolimbic networks that facilitate the prediction of future reinforcement (or punishment) when the right cues are present (Berridge et al. 2009). These may include the activity of dopamine cells in the VTA, but also the activity other structures that may not be sensitive to ghrelin. In this sense, GHSR KO rats or mice may still exhibit sex motivation, but this may be attenuated and the full expression of these behaviors would require intact GHSR signalling.

Although few papers have examined the effects of a short 24 h fast on T concentrations in rats, one does show that testosterone concentrations are lower in rats that are fasted for 24 h or more (Guezennec et al. 1984). While we did not measure plasma T concentrations in male FHH-GHSR$^{m1/Mcwi}$ rats or their WT littermates, LH concentrations in plasma samples obtained from female rats of this same strain are higher in KO rats than concentrations from samples taken from WT female rats (Sabrin 2014). This was true of whether the rats were fasted or had free access to food. Furtthermore, icv ghrelin treatment is associated with decreased LH and T concentrations in male rats (Martini et al. 2006), so one would expect that if anything, male FHH-GHSR$^{m1/Mcwi}$ rats have higher levels of testosterone than WT rats, and that their deficits in sex motivation are not due to lower T concentrations.

In Experiment 2, it is clear that food deprivation had a strong inhibitory effect in sex anticipatory activity, and this inhibitory effect was enhanced by the ghrelin receptor antagonist. We propose that, while the overall effect of the fast was to reduce sex motivation, the effect of ghrelin in the VTA is one of enhancing motivation. This effect is not seen in ad lib fed rats receiving ghrelin because they are already displaying a high level of motivation and ghrelin may not be able to further elevate this. The motivational effect of ghrelin, however, is unmasked by the effect of the antagonist on sex motivation seen in food deprived rats.

At the general level, our data are in agreement with the idea that there are different neural substrates controling appetitive and consummatory reproductive behaviors and these neural substrates differ in their sensitivity to changes in food availability (Beach 1976; Everitt 1990; Schneider et al. 2013). Thus, while Long Evans male rats displayed fewer level changes in anticipation of female rats when food deprived overnight, once in the presence of a receptive female, they displayed few if any deficits in copulatory behaviors. In Syrian hamsters, a solitary rodent species where males forage during restricted periods during the day (dawn and dusk), energy shortages decrease motivational components of sex behaviors without altering sex performance as a trade-off to forage for food (Schneider et al. 2017). In this species these effects are mediated by the activation of neurons that secrete the RFamide-related peptide 3 (RFRP-3), a peptide associated with the inhibition of the reproductive axis (Schneider et al. 2017). Whether this mechanism is also present in rats, and whether ghrelin targets these neurons to decrease sex motivation in fasted male rats remains to be determined. Nevertheless, long periods of food restriction, more severe food deprivation parameters, or increased metabolic demands result in more generalized effects that include deficits in the neural systems controlling sex behaviors and endocrine function (Ammar et al. 2000; Schneider 2004; Woodside et al. 2012). Ghrelin could also play a role under these extreme circumstances potentially through central effects that include the recruitment of signals from both NPY/AGRP cells in the ARC, and RFRP-3 cells in the dorsomedial hypothalamus to suppress gonadotropin release, ultimately resulting in lower circulating testosterone concentrations. Indeed, there are data suggesting that this could be the case (Celik et al. 2016; Farkas et al. 2013; Furuta et al. 2001; Kalra et al. 1988; Kluge et al. 2007). It is also important to mention that there are inter and intra species differences in the susceptibility of appetitive and consummatory reproductive behaviors to nutritional shortages (Blank and Desjardins 1985; Dooley and Prendergast 2012), and these differences may determine to what extent metabolic signals like ghrelin influence reproductive function (Schneider and Deviche 2017).

Finally, while nutritional shortages influence reproductive function and behavior in male and female mammals, males generally continue to reproduce under nutritionally challenging conditions, and their endocrine and reproductive systems are less susceptible to increased energetic demands than the reproductive system of female mammals (Schneider et al. 2012; Wade and Schneider 1992). While most species of male mammals will spend a significant amount of energy finding mates and competing for access to them, female mammals spend more energy investing in parental care (Schneider et al. 2013). Thus, while our experiments provide data related to male rat reproductive behavior, one would predict a more marked effect in both appetitive and consummatory behaviors of female rats. It is likely that given the greater energetic demands associated with reproduction in females, female reproductive behaviors are more sensitive to changes in nutritional signals including those provided by fluctuating ghrelin concentrations. A recent study shows that female sex behavior in mice is also influenced by fasting or chronic food restriction and these effects can be rescued by treatment with ghrelin receptor antagonists (Bertoldi et al. 2011). Whether other aspects of female sexual motivation are affected by ghrelin, and whether these effects are greater in magnitude than the effects observed on males in this experiment, remains to be determined.

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