



Ghrelin and GHS-R1A signaling within the ventral and laterodorsal tegmental area regulate sexual behavior in sexually naïve male mice



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ABSTRACT

In addition to food intake and energy balance regulation, ghrelin mediate the rewarding and motivational properties of palatable food as well as addictive drugs. The ability of ghrelin to regulate reinforcement involves the cholinergic-dopaminergic reward link, which encompasses a cholinergic projection from the laterodorsal tegmental area (LDTg) to the ventral tegmental area (VTA) together with mesolimbic dopaminergic projections from the VTA to the nucleus accumbens (NAc). Recently, systemic ghrelin was shown to regulate sexual behavior and motivation in male mice via dopamine neurotransmission. The present study therefore elucidates the role of ghrelin and ghrelin receptor (GHS-R1A) antagonist treatment within NAc, VTA or LDTg for sexual behavior in sexually naïve male mice. Local administration of the GHSR-1A antagonist, JMV2959, into the VTA or LDTg was found to reduce the preference for female mice, the number of mounts and the duration of mounting as well as to prolong the latency to mount. This was further substantiated by the findings that ghrelin administration into the VTA or LDTg increased the number of mounts and the duration of mounting and decreased the latency to mount. Moreover, ghrelin administered into the LDTg increased the preference for female mice. Accumbal administration of ghrelin increased whereas GHS-R1A antagonist decreased the intake of palatable food, but did not alter sexual behavior. In males exposed to sexual interaction, systemic administration of ghrelin increases whereas JMV2959 decreases the turnover of dopamine in the VTA. These data suggest that ghrelin signaling within the tegmental areas is required for sexual behavior in sexually naïve male mice.

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1. Introduction

Human and rodent studies collectively indicate that the orexigenic peptide ghrelin increases the incentive value of motivated behaviors and has a role in addictive behaviors (for review (Engel and Jerlhag, 2014)). Indeed, rodent studies show that central as well as peripheral ghrelin administration causes accumbal dopamine release in vivo as well as in vitro and that peripheral ghrelin administration causes a focal activation of a network of reward related areas such as the ventral tegmental area (VTA), nucleus accumbens (NAc) and lateral hypothalamus in rats (for review see (Engel and Jerlhag, 2014)). This is further substantiated by preclinical data showing that ghrelin increases whereas ghrelin receptor (GHS-R1A) antagonists decrease alcohol intake, alcohol-induced reward and the motivation to consume alcohol for review see (Engel and Jerlhag, 2014)). These preclinical findings have been translated to human studies showing that the baseline ghrelin levels correlate

with self-reported craving scores in alcohol dependent individuals and that the plasma concentration of active ghrelin are positive correlated to alcohol craving (for review see (Feduccia and Leggio, 2014)). In addition, intravenous administration of ghrelin increases alcohol craving in alcohol-dependent heavy-drinking individuals (for review see (Feduccia and Leggio, 2014)). Furthermore the rewarding properties of addictive drugs are influenced by ghrelin signaling in rats (for review see (Engel and Jerlhag, 2014)). In addition to reward induced by addictive drugs, ghrelin signaling is required for the motivation to consume palatable food as well as food reward (Disse et al., 2010; Egecioglu et al., 2010; Landgren et al., 2011; Skibicka et al., 2011). The ability of ghrelin to regulate reinforcement involves the cholinergic-dopaminergic reward link (Egecioglu et al., 2010; Jerlhag et al., 2009; Skibicka et al., 2011), which encompasses a cholinergic projection from the laterodorsal tegmental area (LDTg) to the VTA and the subsequent dopaminergic projections from the VTA to the NAc.

In addition to food and drug reward the cholinergic-dopaminergic reward link mediates the motivational and rewarding properties of sexual behavior (for review see (Shadiack and Althof, 2008)). Albeit the importance of dopamine for sexual

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behavior has been debated (for review see (Paredes and Agmo, 2004)). Recently we showed that peripheral administration of ghrelin increases whereas genetic or pharmacological suppression of GHS-R1A signaling decreases the sexual behavior in male mice and that this involves dopamine neurotransmission (Egecioglu et al., 2014). The present series of experiments were designed to examine the neuronal circuits underlying the ability of ghrelin signaling to regulate sexual behavior in mice, with focus on the cholinergic-dopaminergic reward link. Thus, the effects of local infusion of ghrelin or the GHS-R1A antagonist, JMV2959, into the NAc, VTA or LDTg on the latency to mount, number of mounts, total sexual interaction time (i.e. sexual interaction) and preference for the opposite sex (reflecting sexual motivation) in male mice were evaluated in the present study.

2. Material and methods

2.1. Animals

Adult post-pubertal age-matched male NMRI mice (B&K Universal AB, Sollentuna, Sweden) and ovariectomized female C57Bl/6N mice were used (B&K Universal AB). All mice were maintained at 20 °C with 50% humidity and a 12/12 h light/dark cycle. Tap water and food (Normal chow; Harlan Teklad, Norfolk, England) were supplied ad libitum, except during the experimental sessions. All experiments were conducted in sexually naïve male mice and each single experiment used an independent set of mice. The Ethics Committee for Animal Experiments in Gothenburg, Sweden approved the studies.

2.2. Guide cannula implantation

To enable the local and bilateral administration, guide cannulas, aiming either at the NAc, VTA or the LDTg, were surgically implanted ($n=8$ per treatment and experimental set up was used). The mice were anesthetized with isofluran (Isofluran Baxter; Univentor 400 Anaesthesia Unit, Univentor Ltd., Zejtun, Malta), placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA, USA) and kept on a heating pad to prevent hypothermia. Xylocain adrenalin (5 µg/ml; Pfizer Inc, New York, USA) was used as local anesthetics and carprofen (5 mg/kg i.p.; Rimadyl®; Astra Zeneca, Gothenburg, Sweden) was used to relieve pain. Two holes for the guide cannulas (stainless steel, length 10 mm, with an o.d./i.d. of 0.6/0.45 mm) and one for the anchoring screw were drilled. The coordinates (Franklin and Paxinos, 1997) relative to bregma, lateral to the midline and below the brain surface were: NAc +1.5 mm, ±0.8 mm, −1.0 mm, VTA −3.4 mm, ±0.5 mm, −1.0 mm, LDTg −5.0 mm, ±0.5 mm, −1.0 mm. The guide cannulas and screw were stabilized with dental cement (DENTALON® plus; AgnTho's AB, Lidingö, Sweden). The mice were kept in individual cages (Seal-safe IVC 2L, 365 × 207 × 140 mm) and were allowed to recover for three days before the experiment. At the time of the experiment, a cannula for drug administration was inserted and extended another 3.7 mm, 3.8 mm or 2.2 mm ventrally beyond the tip of the guide cannula, aiming at the NAc, VTA and LDTg respectively (Franklin and Paxinos, 1997). The drug, at a volume of 0.5 µl, was administered over one minute; the cannula was left in place for another minute and the cannula was then retracted (5 µl Kloehe, microsyringe; Skandinaviska Genetec AB, V. Frölunda, Sweden). The injection sites were verified following the termination of the experiment, and only animals with correct placements were included in the statistical analysis.

2.3. Ovariectomy of female mice

Female C57Bl/6N mice, aged 10 weeks at arrival, were anaesthetized with a mixture of ketamine (Ketalar 10 mg/ml, Pfizer, Apoteket AB, Gothenburg, Sweden) and xylazine (Rompun Vet 20 mg/ml, Bayer Animal Health, Apoteket AB, Gothenburg, Sweden), ovariectomized via a midline incision and subsequently allowed to recover in temperate cages in groups of eight. Female mice were brought into oestrus by giving sequential injection of 10 and 5 µg of estradiol benzoate 48 and 24 h before the test and 50 µg of progesterone 4 h before the tests. Female mice were used 4–7 h after progesterone injections.

2.4. Drugs

Acylated rat ghrelin (Bionuclear; Bromma, Sweden) was diluted in vehicle solution (Ringer) for local administration into the NAc, VTA and LDTg. The selected dose of ghrelin (1 µg per side) for injections into the VTA and LDTg was based on previous studies showing that local administration of this dose into either the VTA or LDTg causes a locomotor stimulation, accumbal dopamine release as well as increases alcohol intake (Jerlhag et al., 2007, 2009). The dose of ghrelin (1 µg per side) given into the NAc was based on a dose response study presented herein (Supplementary information 1). Ghrelin was always administered ten minutes prior to the experiment. The selected doses of the GHS-R1A antagonist, JMV2959 (provided by Æterna Zentaris GbmH, Frankfurt am Main, Germany), administered into the NAc, VTA and LDTg twenty minutes prior to the initiation of the experiment, were based on dose response studies investigating locomotor activity responses (Supplementary information 1). Radioligand binding studies in vitro have also established that JMV2959 is a selective and competitive GHS-R1A antagonist (Moulin et al., 2007). JMV2959 was dissolved in vehicle (Ringer).

2.5. Locomotor activity experiments

Locomotor activity tests were conducted to identify doses of JMV2959 for local bilateral administration into the NAc, VTA or LDTg and for bilateral ghrelin into the NAc to be used in the sexual behavior tests (Supplementary information 1). Locomotor activity was recorded as described previously (Jerlhag et al., 2007, 2009) (for detailed information see supplementary information 1).

JMV2959 was administered bilaterally into the NAc (5 µg, 10 µg or 20 µg per side), VTA (5 µg or 10 µg side) or LDTg (10 µg or 20 µg per side). Ghrelin (0.5 µg, 1 µg or 2 µg per side) was administered bilaterally into the NAc in mice and locomotor activity explored. The mice used in the dose response studies received one treatment only ($n=8$ per treatment) and was only subjected to one experimental trial.

2.6. Preference for female mouse (sexual motivation test)

A plastic cage (50 × 39 × 41 cm, SmartStore Classic; Hammarplast Consumer AB, Gothenburg, Sweden) with holes cut out for cages (10 × 10 × 10 cm) for incentive animals at opposing sides along the long side of the arena was used as previously described (Egecioglu et al., 2014). An area of 14 × 10 cm immediately adjacent to the cages with the incentive animals was designated as the male or female incentive zone. Each test mouse was allowed to habituate to the testing area for 10 min on 2 consecutive days before the experiment. One hour before initiating the experiment a dummy cannula was carefully inserted and retreated into the guide cannula to remove clotted blood and to hamper spreading depression. Immediately prior to the test, mice were allowed to habituate to the arena for 15 min, whereupon cages with novel stimulus ani-

Table 1

Dose selection for local administration in nucleus accumbens (NAc), ventral tegmental area (VTA) or laterodorsal tegmental area (LDTg).

| | Ghrelin (μg per side) | JMV2959 (μg per side) |
|------|-----------------------------------|-----------------------------------|
| NAc | 1 | 10 |
| VTA | 1 | 5 |
| LDTg | 1 | 10 |

mals, one ovariectomized oestrus female and one male were placed at the openings allowing olfactory and visual cues, but not contact, between the animals. Placement of male and female incentive animals was randomized and the arena and mesh barriers were cleaned with water followed by alcohol solution (10%) between the individual tests. Movement patterns were recorded for 15 min and an observer blind to the treatment of each individual mouse subsequently analyzed time spent in the female and male incentive zone. Preference for female mouse is defined as the time at female incentive zone divided by the total interaction time.

Prior to the preference test ghrelin or an equal volume of vehicle was administered bilaterally into the NAc, VTA or LDTg (see Table 1). In separate mice JMV2959 or an equal volume of vehicle was administered bilaterally into the NAc, VTA or LDTg (see Table 1). The experiments were conducted with three weeks apart and the same females were used for three tests and other females were used for the other three series of experiments.

2.7. Latency to mount, number of mounts and total sexual interaction time (i.e. sexual interaction test)

Test for sexual behavior was performed as previously described (Egecioglu et al., 2014). In brief, it was conducted by placing an ovariectomized female mouse in artificial oestrus in the home cage of the male mouse. The females were exposed to sexual interaction before the present test and each female was only used twice per oestrus induction. One hour before initiating the experiment a dummy cannula was carefully inserted and retreated into the guide cannula to remove clotted blood and to hamper spreading depression. In all tests, male test mice were allowed to interact with the female for 20 min and the interaction was videotaped for subsequent analysis of behavior by an observer blind to the treatment of individual mice. The number of mounts, the total sexual interaction time as well as latency to mounting was investigated. Mounting, i.e. pelvic thrusting is the basic unit of copulatory behavior (for review see (Paredes and Agmo, 2004)). We also scored for intromission, which is defined as male pelvic thrust with a stable frequency continuously for more than six seconds causing an elevation of the female's anogenital area (Kikusui, 2013; Paredes and Agmo, 2004). However, there was not enough intromissions in these experiments for statistical analysis. All the sexual behavior experiments were screened for ejaculation behavior, but ejaculation was not observed in these tests. Supportively, male mice do not always achieve ejaculation within a 20 min assay (McGill, 1962; Raskin et al., 2009).

Prior to the interaction test, ghrelin or an equal volume of vehicle was administered bilaterally into the NAc, VTA or LDTg (see Table 1). In separate mice JMV2959 or an equal volume of vehicle was administered bilaterally into the NAc, VTA or LDTg (see Table 1). These experiments were conducted with three weeks apart and the same females were only used for these three subsequent tests.

2.8. Food intake experiments

To verify that the selected doses of ghrelin or JMV2959 administered into the NAc were of physiological relevance intake of chow

and peanut butter was investigated using the same doses. All mice were allowed to familiarize to the taste of peanut butter for one week before the test. The effect of ghrelin (1 μg per side) or vehicle (Ringer) administration bilaterally into the NAc on peanut butter and chow intake was investigated in the home cage for two hours.

In another set of mice, the effect of local administration of JMV2959 (10 μg per side) or vehicle (Ringer) bilaterally into the NAc on peanut butter and chow intake was investigated in the home cage for two hours.

2.9. Biochemical analysis of tissue samples

The effect of peripheral ghrelin (0.33 mg/kg) or JMV2959 (6 mg/kg) treatment on dopamine ((DOPAC + HVA)/DA) turnover in the VTA was investigated in mice that had undergone sexual interaction (interaction data published previously (Egecioglu et al., 2014)). The mice were decapitated directly, the brains were removed and the VTA was rapidly dissected out on a cold glass plate, and kept frozen at -80°C until analysis. Dissected brain tissue samples were homogenized by ultrasound homogenization (Sonifier Cell Disruptor B30, Branson Sonic Power Co. Danbury, CT, USA) in a solution of 0.1 M perchloric acid, 5.37 mM EDTA and 0.65 mM glutathione. After centrifugation (10,000 rpm, 5°C , 10 min) the supernatant was collected and analysed for dopamine and the metabolites DOPAC and HVA using a split fraction HPLC-ED system. Dopamine was analysed on an ion-exchange column (Nucleosil, 5 μm SA 100 A, 150×2 mm, Phenomenex, Torrance, CA, USA) with a mobile phase consisting of 13.3 g citric acid, 5.84 g NaOH, 40 mg EDTA, and 200 ml methanol in distilled water to a total volume of 1000 ml. DOPAC and HVA and 5-HIAA were analysed on a reverse phase column (Nucleosil, 3 μm , C18, 100 A, 50×2 mm, Phenomenex) with a mobile phase consisting of 11.22 g citric acid, 3.02 g dipotassium phosphate, 40 mg EDTA, and 60 ml methanol in distilled water to a total volume of 1000 ml. The electrochemical detections were performed by two amperometric detectors (Waters 460) and the currents were recorded with the Dionex Chromeleon software package (Dionex, Sunnyvale, CA, USA).

2.10. Statistics

Locomotor activity data was evaluated by a one-way ANOVA followed by Bonferroni post-hoc tests. Effects of local administration of ghrelin or JMV2959 compared to vehicle treatment on preference for female mice, sexual interaction, food intake and dopamine turnover were analyzed with an unpaired *t*-test. Data are presented as mean \pm SEM. A probability value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Effects of administration of ghrelin or JMV2959 into the NAc on female preference as well as on the latency to mount, number of mounts and total sexual interaction time in sexually naïve male mice

Accumbal ghrelin treatment did not alter the preference for the female mouse compared to vehicle treatment ($P = 0.2763$; unpaired *t*-test; $n = 8$ in each group; Fig. 1A). Ghrelin did not affect the total interaction time (male + female interaction) compared to vehicle treatment ($P = 0.8652$; unpaired *t*-test; Fig. 1B).

Ghrelin administration into NAc did not affect the latency for sexual interaction with female mouse compared to vehicle treatment ($P = 0.6035$; unpaired *t*-test; $n = 7$ for Veh; $n = 8$ for Ghrelin; Fig. 1C). Moreover, ghrelin did neither alter the number of mounts ($P = 0.5071$; unpaired *t*-test; Fig. 1D) nor the mounting duration with female mouse ($P = 0.4725$; unpaired *t*-test; Fig. 1E).

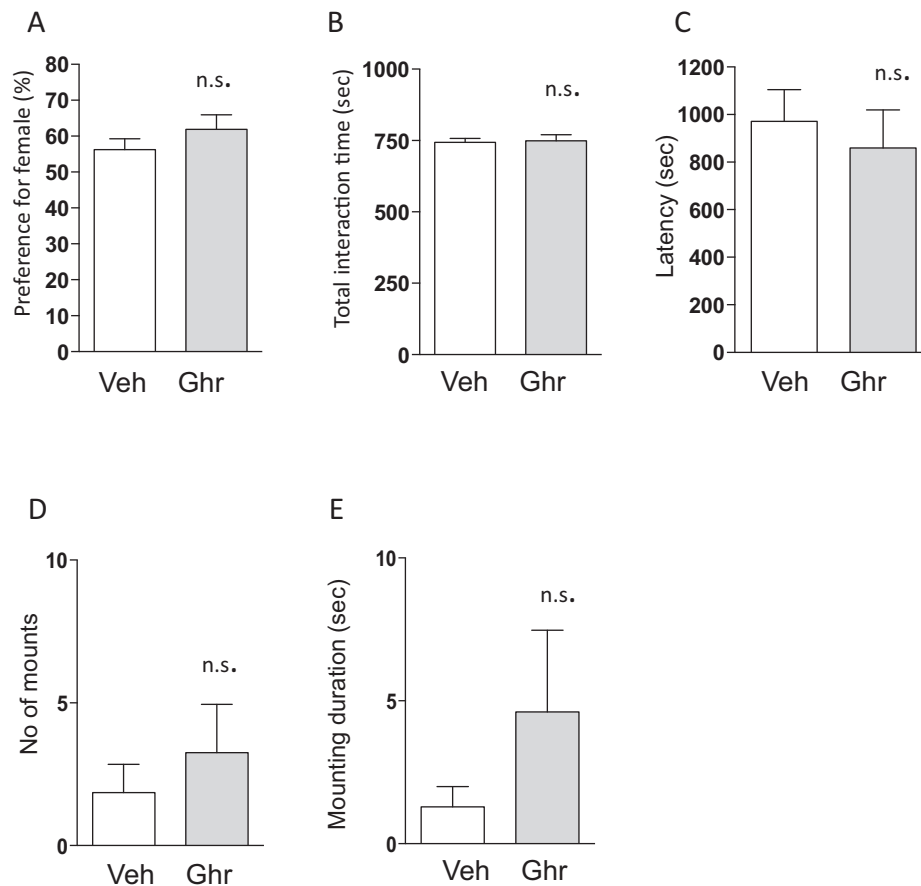


Fig. 1. Local administration of ghrelin into the NAc on the preference for female mouse as well as sexual behavior in sexually naïve male mice. (A) Accumbal ghrelin (Ghr) treatment did not alter the preference for the female mouse compared to vehicle (Veh) treatment ($n = 8$ in each group). (B) Ghrelin did not affect the total interaction time (male + female interaction) compared to vehicle treatment. (C). Ghrelin administration into NAc did not affect the latency for sexual interaction with female mouse compared to vehicle treatment ($n = 7$ for Veh; $n = 8$ for Ghrelin). Accumbal ghrelin did neither alter the number of mounts (D) nor the mounting duration (E) with female mouse. n.s. $P > 0.05$. Data analysed with an unpaired t -test. All values represent mean \pm SEM.

JMV2959, the GHS-R1A antagonist, treatment into the NAc did not affect the preference for female mouse compared to vehicle treatment ($P = 0.3629$; unpaired t -test; $n = 8$ in each groups; Fig. 2A). JMV2959 did not affect the total interaction time compared to vehicle treatment ($P = 0.7603$; unpaired t -test; Fig. 2B).

Accumbal JMV2959 administration did not alter the latency for sexual interaction with female mouse compared to vehicle treatment ($P = 0.2989$; unpaired t -test; $n = 8$ in each group; Fig. 2C). Local JMV2959 treatment did not affect the number of mounts ($P = 0.1502$; unpaired t -test; Fig. 2D) or the mounting duration with female mouse ($P = 0.1890$; unpaired t -test; Fig. 2E).

3.2. Effects of ghrelin or JMV2959 administration into the VTA on female preference as well as on the latency to mount, number of mounts and total sexual interaction time in sexually naïve male mice

Local and bilateral administration of ghrelin into the VTA did neither affect the preference for the female mouse compared to vehicle treatment ($P = 0.2171$; unpaired t -test; $n = 8$ in each group; Fig. 3A) nor the total interaction time (male + female interaction) compared to vehicle treatment ($P = 0.6183$; unpaired t -test; Fig. 3B).

Ventral tegmental ghrelin treatment significantly reduced the latency for sexual interaction with female mouse compared to vehicle treatment ($P = 0.0189$; unpaired t -test; $n = 7$ for Veh; $n = 8$ for Ghrelin; Fig. 3C). Moreover, ghrelin significantly increased the number of mounts ($P = 0.0448$;

unpaired t -test; Fig. 3D) and increased the mounting duration with female mouse ($P = 0.0405$; unpaired t -test; Fig. 3E).

JMV2959, the GHS-R1A antagonist, treatment into the VTA significantly decreased the preference for female mouse compared to vehicle treatment ($P = 0.0096$; unpaired t -test; $n = 8$ in each group; Fig. 4A). JMV2959 did not affect the total interaction time compared to vehicle treatment ($P = 0.6723$; unpaired t -test; Fig. 4B).

Ventral tegmental JMV2959 administration significantly increased the latency for sexual interaction with female mouse compared to vehicle treatment ($P = 0.0048$; unpaired t -test; $n = 8$ for vehicle; $n = 7$ for JMV; Fig. 4C). Moreover, JMV2959 significantly decreased the number of mounts ($P = 0.0342$; unpaired t -test; Fig. 4D) as well as decreased the mounting duration with female mouse ($P = 0.0396$; unpaired t -test; Fig. 4E).

3.3. Effects of local and bilateral administration of ghrelin or JMV2959 into the LDTg on female preference as well as on the latency to mount, number of mounts and total sexual interaction time in sexually naïve male mice

Local ghrelin treatment into the LDTg significantly increased the preference for the female mouse compared to vehicle treatment ($P = 0.0115$; unpaired t -test; $n = 8$ for vehicle and $n = 7$ for ghrelin; Fig. 5A). Ghrelin did not affect the total interaction time (male + female interaction) compared to vehicle treatment

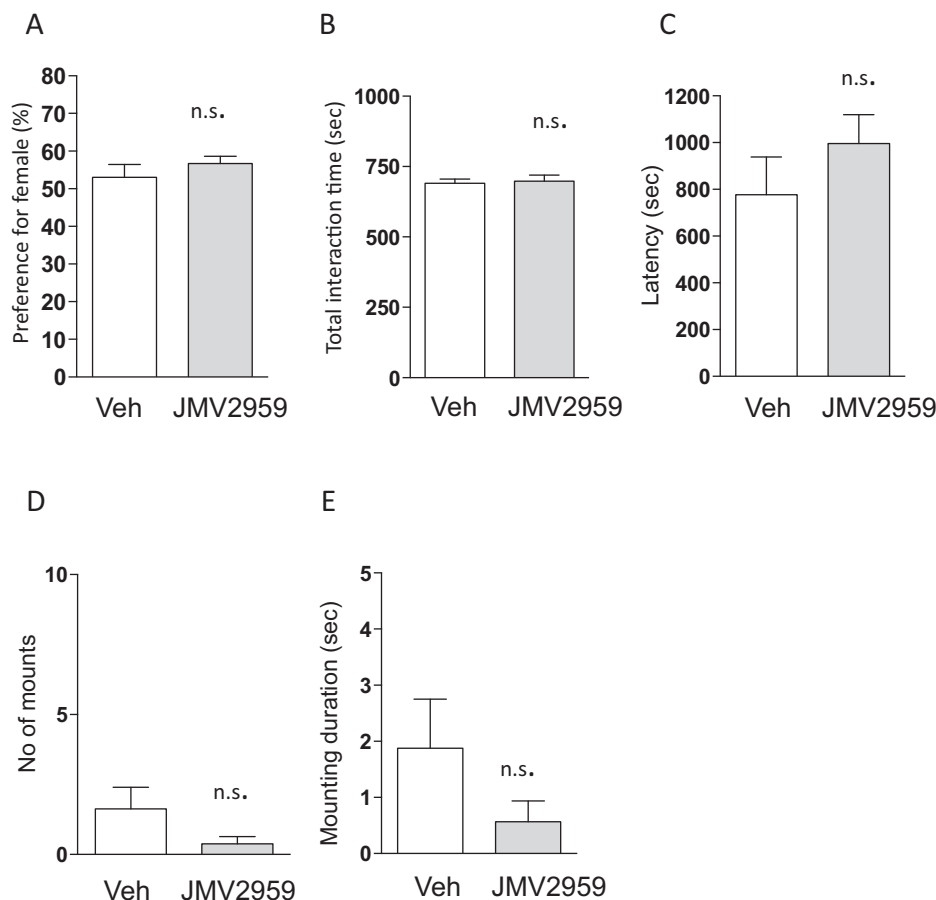


Fig. 2. Local administration of the GHSR-1A antagonist into the NAC on the preference for female mouse as well as sexual behavior in sexually naïve male mice.

(A) Accumbal JMV2959 (JMV) treatment did not alter the preference for the female mouse compared to vehicle (Veh) treatment ($n=8$ in each group). (B) JMV2959 did not affect the total interaction time (male + female interaction) compared to vehicle treatment. (C) JMV2959 administration into NAC did not affect the latency for sexual interaction with female mouse compared to vehicle treatment ($n=8$ in each group). Accumbal JMV2959 did neither alter the number of mounts (D) nor the mounting duration (E) with female mouse. n.s. $P>0.05$. Data analyzed with an unpaired t -test. All values represent mean \pm SEM.

($P=0.0849$; unpaired t -test; Fig. 5B). Local and bilateral ghrelin infusion into the LDTg significantly reduced the latency for sexual interaction with female mouse compared to vehicle treatment ($P=0.0023$; unpaired t -test; $n=8$ in each group; Fig. 5C). Moreover, ghrelin significantly increased the number of mounts ($P=0.0331$; unpaired t -test; Fig. 5D) and increased the mounting duration with female mouse ($P=0.0372$; unpaired t -test; Fig. 5E).

JMV2959, the GHS-R1A antagonist, treatment into the LDTg significantly decreased the preference for female mouse compared to vehicle treatment ($P=0.0044$; unpaired t -test; $n=8$ for JMV2959 and $n=7$ for vehicle; Fig. 6A). JMV2959 did not affect the total interaction time compared to vehicle treatment ($P=0.9378$; unpaired t -test; Fig. 6B).

JMV2959 administration into the LDTg significantly increased the latency for sexual interaction with female mouse compared to vehicle treatment ($P=0.0020$; unpaired t -test; $n=8$ in each group; Fig. 6C). Moreover, JMV2959 significantly decreased the number of mounts ($P=0.0141$; unpaired t -test; Fig. 6D) as well as decreased the mounting duration with female mouse ($P=0.0414$; unpaired t -test; Fig. 6E).

Sexual interaction is lower in mice following local administrations, especially in the LDTg, than compared to systemic injections. This is most likely due to alterations in behavior by surgical implantations. However, the effect of drug is always compared to local administration of vehicle solution rather than peripheral administration.

3.4. Effects of ghrelin or JMV2959 locally administered into NAC on chow and peanut butter intake

Accumbal ghrelin treatment significantly increased the intake of peanut butter compared to vehicle treatment ($P=0.0420$; $n=8$ in each group; Supplementary Fig. 1A). Ghrelin did not affect chow intake compared to vehicle treatment ($P=0.7415$; Supplementary Fig. 1B).

Local administration of JMV2959 into the NAC significantly decreased peanut butter intake compared to vehicle treatment ($P=0.0305$; $n=8$ in each group; Supplementary Fig. 1C). JMV2959 did not affect the intake of chow compared to vehicle treatment ($P=0.2633$; Supplementary Fig. 1D).

3.5. Effects of ghrelin or the GHS-R1A antagonist on dopamine turnover in the VTA following sexual interaction

Compared to vehicle treatment, systemic administration of ghrelin ($P=0.0332$, Supplementary Fig. 2A) decreased, whereas JMV2959 ($P=0.0075$, Supplementary Fig. 2B) increased the turnover of dopamine ((DOPAC + HVA)/DA) in the VTA in male mice exposure to a female in oestrus in the sexual interaction test.

4. Discussion

The present series of experiments show that ghrelin signaling within both the VTA and LDTg, two of the main regions of the

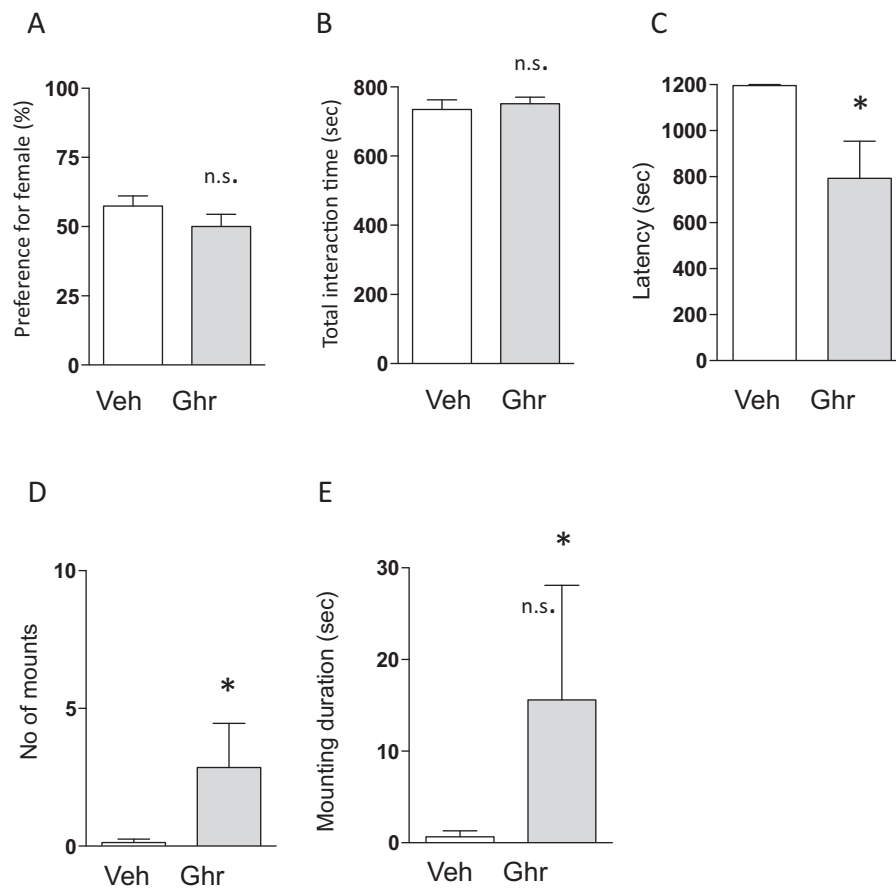


Fig. 3. Local administration of ghrelin into the VTA on the preference for female mouse as well as sexual behavior in sexually naïve male mice.

(A) Ghrelin (Ghr) treatment into the VTA did not alter the preference for the female mouse compared to vehicle (Veh) treatment ($n = 8$ in each group). (B) Ghrelin did not affect the total interaction time (male + female interaction) compared to vehicle treatment. (C) Ghrelin administration into VTA decreased the latency for sexual interaction with female mouse compared to vehicle treatment ($n = 7$ for Veh; $n = 8$ for Ghrelin). Ventral tegmental ghrelin increased the number of mounts (D) as well as the mounting duration (E) with female mouse compared to vehicle treatment. * $P < 0.05$ and n.s. $P > 0.05$. Data analyzed with an unpaired t -test. All values represent mean \pm SEM.

cholinergic-dopaminergic reward link, regulates sexual behavior in mice. On the contrary, ghrelin and GHS-R1A antagonism within the NAc does not affect sexual behavior in male mice although it modulates the intake of palatable food. Collectively, these data suggest that ghrelin and its receptor in the LDTg and VTA are required for normal sexual behavior in sexually naïve male mice. In addition, the ghrelin effects on palatable food intake and sexual behavior appear to be mediated by different parts of the reward circuitries in the brain.

In line with the hypothesis that dopamine is the main mediator of rewarding and motivational behaviors, previous pharmacological studies have shown that reduced dopaminergic activity attenuates whereas enhanced dopaminergic activity facilitates sexual behavior in rodents (for review see (Shadiack and Althof, 2008)). For instance, systemic administration of D2 antagonists or a D1 antagonist decreased sexual behavior and reduce the number of anticipatory level changes (Pfaus and Phillips, 1991). It should however be mentioned that there are contradicting findings regarding the role of dopamine in sexual behavior (for review see (Paredes and Agmo, 2004)). As described above, we and others, have shown that dopamine mediates the rewarding effects of ghrelin induced by food and addictive drugs (for review see (Engel and Jerlhag, 2014)). In addition, we recently found that treatment with L-dopa, the precursor of dopamine, restored the decrease preference for female mouse following GHS-R1A antagonist treatment (Egecioglu et al., 2014), suggesting that ghrelin modulates sexual reward and motivation in male mice by modulation of dopamine

neurotransmission. This notion was supported in the current study where it was shown that systemic administration of ghrelin increases whereas JMV2959 decreases the turnover of dopamine in the VTA in male mice subjected to sexual interaction. Given that ghrelin injection into the VTA increases accumbal dopamine release in mice (Jerlhag et al., 2007) and that sexual behavior, such as copulation, increases NAc dopamine release (Damsma et al., 1992; Mas et al., 1990; Pleim et al., 1990), it might be hypothesized that ghrelin mediated sexual behavior involves NAc dopamine release. However, ventral tegmental dopamine neurons project to other areas including the medial preoptic area (MPOA) (Reynolds et al., 2006), raising the possibility that ghrelin signaling within the VTA regulates sexual behavior via dopamine release in the MPOA. Indeed, sexual interaction increase cFos expression in the MPOA in rats (McCarthy et al., 1997; Struthers, 2001) and pharmacological manipulation of MPOA-dopaminergic neurotransmission as well as lesion of MPOA impairs sexual interaction as well as preference for a female rat (for review see (Everitt, 1990; Hull and Dominguez, 2007)). The role of these VTA-dopamine projections for ghrelin mediated sexual behavior should be explored in upcoming studies.

In the present study we found that local administration of JMV2959 into the VTA reduced the preference for female mouse, the number of mounts and the duration of mounting as well as enhance the latency to mount. In addition, we found that ghrelin administration into the VTA decreases the latency to mount as well as increase the number of mounts and the duration of mounting. In support for an important role of the VTA that expresses

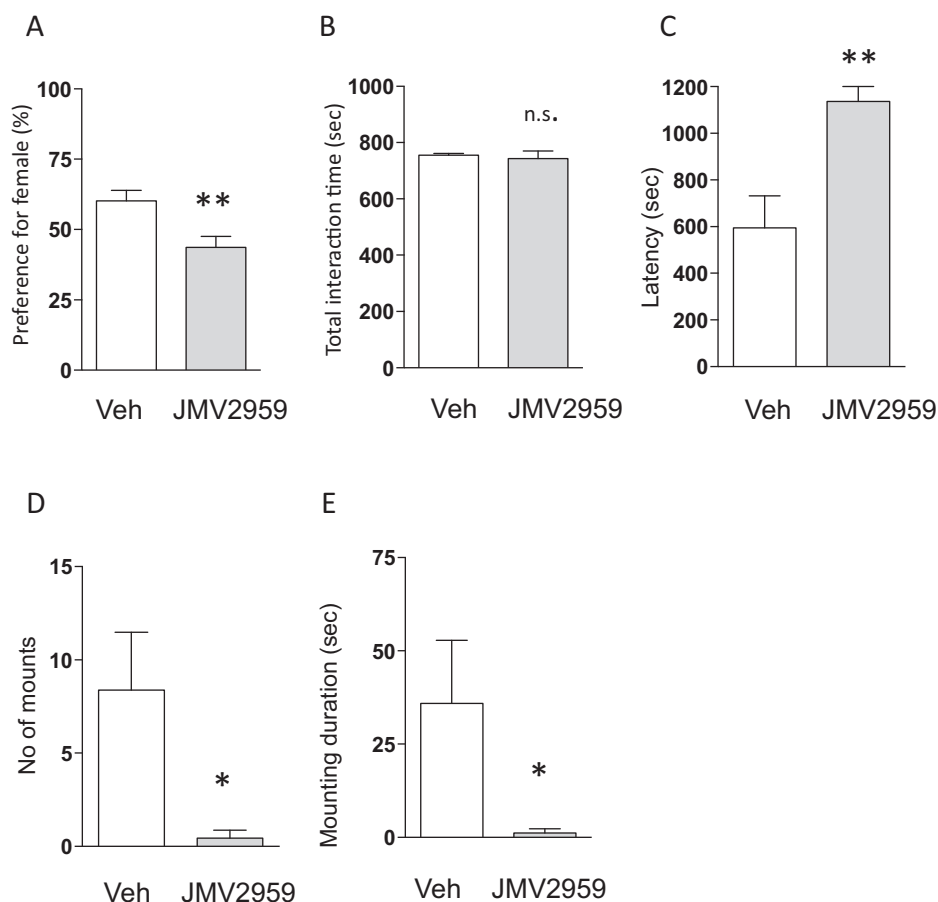


Fig. 4. Local administration of the GHSR-1A antagonist into the VTA on the preference for female mouse as well as sexual behavior in sexually naïve male mice.

(A) Ventral tegmental JMV2959 (JMV) treatment decreased the preference for the female mouse compared to vehicle (Veh) treatment ($n = 8$ in each group). (B) JMV2959 did not affect the total interaction time (male + female interaction) compared to vehicle treatment. (C) JMV2959 administration into VTA increased the latency for sexual interaction with female mouse compared to vehicle treatment ($n = 8$ for vehicle, $n = 7$ for JMV). Ventral tegmental JMV2959 reduced the number of mounts (D) and the mounting duration (E) with female mouse. * $P < 0.05$, ** $P < 0.01$ and n.s. $P > 0.05$. Data analyzed with an unpaired t -test. All values represent mean \pm SEM.

GHS-R1A (for review see (Engel and Jerlhag, 2014)), in sexual behavior are the findings showing that sexual behavior increases firing of ventral tegmental dopamine neurons (Hernandez-Gonzalez et al., 1997) as well as VTA-cFos expression (Lopez and Ettenberg, 2002). In addition, copulation is facilitated by electrical stimulation of the VTA (Markowski and Hull, 1995) and lesions of the VTA reduced sexual behavior (for review see (Hull et al., 2006)). The importance of ghrelin and GHS-R1A signaling within the VTA for motivated behaviors are indicated by previous studies showing that ventral tegmental alterations of ghrelin sensitive mechanisms effects the consumption, the motivation to consume as well as the rewarding properties of alcohol and of palatable food (Egecioglu et al., 2010; Jerlhag et al., 2009; Skibicka et al., 2011). The present *ex vivo* data show that peripheral treatment with ghrelin decrease, whereas JMV2959 increases the turnover of dopamine in the VTA of male mice exposed to sexual interaction. Taken together with the findings showing that the somatodendritic release of dopamine within the VTA activates inhibitory postsynaptic D2 receptors on dopaminergic neurons (Lacey et al., 1987) and that the sensitivity of the dopamine neurons in the VTA are altered by the ability of GHS-R1A to heterodimerize with dopamine D1 and D2 receptors in the VTA (Jiang et al., 2006; Kern et al., 2012), it could be hypothesized that the altered ventral tegmental dopamine levels by ghrelin/JMV2959 may, via this heterodimerization, regulate sexual behavior in naïve male mice.

We showed that local LDTg infusion of ghrelin increases whereas GHSR-1A antagonist decreases the preference for female

mouse as well as sexual behavior in sexually naïve male mice, suggesting that LDTg is important for ghrelin mediated sexual behavior. Supportively, lesion of the cholinergic projections to the VTA decreases sexual interaction and preference for a female in sexually naïve, but not in experienced, male rats (Kippin and van der Kooy, 2003). A role for ghrelin signaling within the LDTg, where GHS-R1A are expressed on cholinergic neurons projecting onto VTA-dopamine neurons (Dickson et al., 2010), for reinforcement regulation is further supported by the data findings that ghrelin administration into the LDTg increases alcohol intake in mice (Jerlhag et al., 2009) as well as induces a concomitant release of VTA-acetylcholine and NAc-dopamine (Jerlhag et al., 2012). However, LDTg project to other areas such a dorsal striatum and substantia nigra (Mesulam et al., 1983), raising the possibility that ghrelin regulated sexual behavior include projection also to these areas.

The present study shows that ghrelin signaling within the VTA as well as LDTg regulates sexual behavior in sexually naïve male mice. However, the possibility that other brain areas orchestrate ghrelin mediated male sexual behavior should be considered and explored. Indeed, local ghrelin administration into the MPOA, expressing GHSR-1A for review see (Engel and Jerlhag, 2014), increases wakefulness as well as food intake (Szentirmai et al., 2007) and the projection from the MPOA to the VTA regulates reward induced by addictive drugs (Tobiansky et al., 2013). Activation of amygdala, another area targeted by ghrelin (Alvarez-Crespo et al., 2012; Hansson et al., 2014), induces sexual behavior in male hamsters

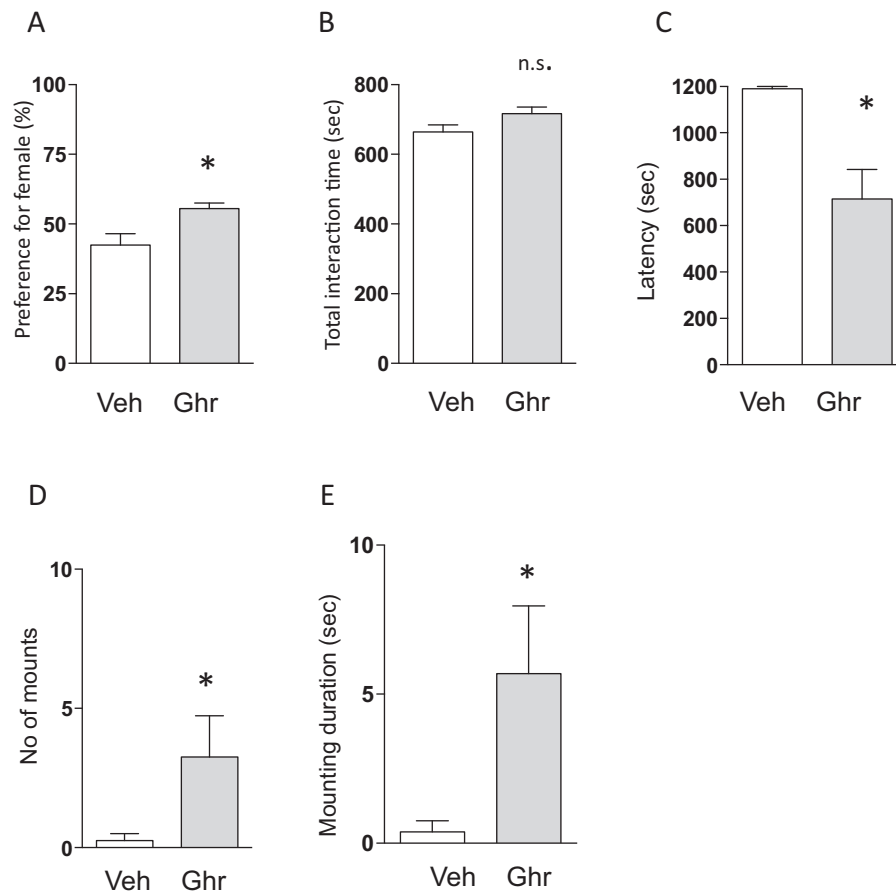


Fig. 5. Local administration of ghrelin into the LDTg on the preference for female mouse as well as sexual behavior in sexually naïve male mice.

(A) Ghrelin (Ghr) treatment into the LDTg increased the preference for the female mouse compared to vehicle (Veh) treatment ($n=6$ for Veh; $n=7$ for Ghrelin). (B) Ghrelin did not affect the total interaction time (male + female interaction) compared to vehicle treatment. (C). Ghrelin administration into VTA decreased the latency for sexual interaction with female mouse compared to vehicle treatment ($n=8$ in each group). Ventral tegmental ghrelin increased the number of mounts (D) as well as the mounting duration (E) with female mouse compared to vehicle treatment. * $P<0.05$, ** $P<0.01$ and n.s. $P>0.05$. Data analyzed with an unpaired t -test. All values represent mean \pm SEM.

(Lehman et al., 1983) and sexual motivation in rats (van Furth et al., 1995). Furthermore, lesions of paraventricular nucleus of hypothalamus, which expresses GHSR-1A and regulate ghrelin mediated hedonic food intake (for review see (Egicioglu et al., 2011)), impair copulation as well as erections in rats (Liu et al., 1997). The present study reports variability in sexual behavior between different batches of vehicle treated mice raising the possibility that this might influence the obtained results. However, this appears less likely since the present data are in line with a previous study showing that ghrelin increases whereas JMV2959 decreases sexual behavior in naïve male mice (Egicioglu et al., 2014) and variability in baseline behavior is commonly observed.

Albeit previous studies have shown that NAc infusions of a D2 antagonist increased the latency to copulate (Pfaus and Phillips, 1991), the present study showed that accumbal ghrelin or GHS-R1A antagonist treatment did not alter sexual behavior in sexually naïve male mice. The possibilities that accumbal ghrelin or JMV2959 infusion does not alter dopaminergic neurotransmission or that accumbal dopamine is of less importance for sexual behavior should therefore be considered. Ghrelin is traditionally known to regulate homeostatic food intake via hypothalamic GHSR-1A (for review see (Egicioglu et al., 2011)). While previous findings show that ghrelin targets the VTA, rather than the NAc, to regulate conditioned place preference for, operant self administration and intake of sucrose and palatable food in rodents (Egicioglu et al., 2010; Skibicka et al., 2011, 2012), we now show that the intake of palatable food is also regulated by GHS-R1A in the NAc.

This is further substantiated by the findings showing that ghrelin administration into the NAc increase chow intake in situations where no palatable food is available (Naleid et al., 2005; Skibicka et al., 2011). In a choice situation of chow and palatable food we show that ghrelin and JMV2959 did not alter chow intake, but alters peanut butter consumption. Collectively, these data indicate that the ability of the orexigenic peptide to regulate the intake of food, food reward and the motivation to consume food or palatable food is complex and involves GHS-R1A within several reward nodes.

In the present study sexually naïve mice was used since it previously was shown that lesion of the cholinergic projections decreases sexual behavior in sexually naïve, but not in experienced, male rats (Kippin and van der Kooy, 2003). It should however be mentioned that different mechanisms may regulate sexual behavior in naïve versus experienced mice. In the present study the mice did not display intromission. Given that we previously showed that systemic administration of ghrelin increased whereas JMV2959 decreased mounting as well as intromission (Egicioglu et al., 2014), we suggest alterations in sex behavior is due to local injections. However, another tentative explanation might be reduced arousal in sexually naïve compared to experience mice as shown previously (for review see (Paredes and Agmo, 2004)). Previous studies report a facilitation of sexual behavior after dopamine stimulation (for review see (Paredes and Agmo, 2004)), suggesting that the ability of ghrelin to increase the sexual interaction is due to the obtained hyperlocomotion. However, this appears less likely

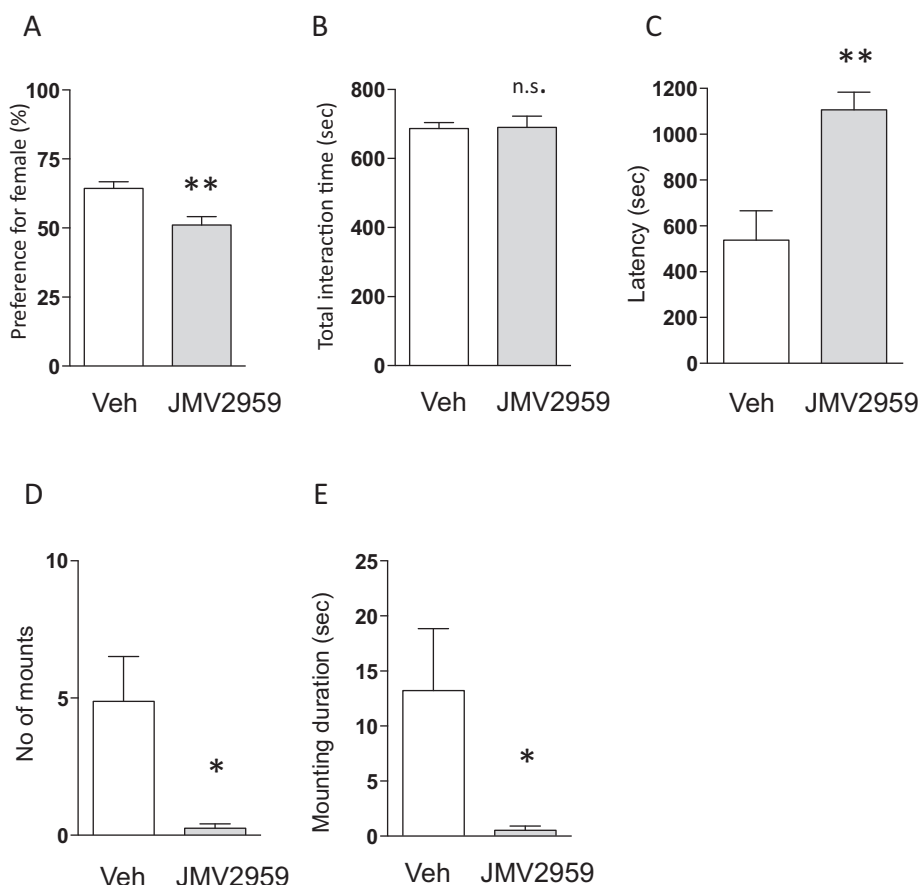


Fig. 6. Local administration of the GHSR-1A antagonist into the LDTg on the preference for female mouse as well as sexual behavior in sexually naïve male mice. (A) JMV2959 (JMV) treatment into the LDTg decreased the preference for the female mouse compared to vehicle (Veh) treatment ($n=7$ for vehicle, $n=8$ for ghrelin). (B) JMV2959 did not affect the total interaction time (male + female interaction) compared to vehicle treatment. (C). JMV2959 administration into LDTg increased the latency for sexual interaction with female mouse compared to vehicle treatment ($n=8$ in each group). Intra-LDTg JMV2959 reduced the number of mounts (D) and the mounting duration (E) with female mouse compared to vehicle treatment. * $P<0.05$, ** $P<0.01$ and n.s. $P>0.05$. Data analyzed with an unpaired t -test. All values represent mean \pm SEM.

since the selected doses of JMV2959 have no effect on motor behavior and that intra-VTA administration of ghrelin increases the intake of peanut butter as well as induced a locomotor stimulation (Egecioglu et al., 2010).

An imaging study show that individuals with compulsive sexual behavior, with a high degree of desire towards visual sex cues, display a more pronounced activation of reward related areas including the ventral striatum, dorsal anterior cingulate and amygdala compared to healthy volunteers (Voon et al., 2014). Given that GHS-R1A antagonism reduces the incentive salience of motivated behaviors, including sexual behavior, it may be proposed that ghrelin-responsive circuits may serve as a novel pharmacological target for treatment of addictive behaviors such as alcohol use disorder and sexual addiction.

The present study is the first to show that ghrelin signaling within the VTA and LDTg regulates sexual behavior, whereas accumbal GHS-R1A modulate the intake of palatable food in mice. In support are the recent data showing that peripheral ghrelin administration increases whereas suppressed ghrelin signaling decreases sexual behavior in male mice (Egecioglu et al., 2014). Collectively, these data indicate that there is a diverse albeit strong overlap between ghrelin activated brain networks involved in the regulation of food hedonics and sexual behavior. In summary, present data therefore contributes to a further understating the role of appetite regulatory peptides in motivation and reinforcement.

Authors contribution

LPG designed and performed the hands on work, analyzed data, wrote the manuscript; EE, LW, ES revised the content and contributed to the conception; EJ designed the study, contributed to the conception and interpretation, managed literature search, analyzed and undertook statistical analysis and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

EJ has for another project received financial support from the NovoNordisk Foundation. This does not alter the authors' adherence to any of the journals policies on sharing data and materials. The remaining authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.psyneuen.2015.09.009>.

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