Central administration of ghrelin alters emotional responses in rats: behavioural, electrophysiological and molecular evidence

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Abstract

The orexigenic and pro-obesity hormone ghrelin targets key hypothalamic and mesolimbic circuits involved in energy balance, appetite and reward. Given that such circuits are closely integrated with those regulating mood and cognition, we sought to determine whether chronic (>two week) CNS exposure to ghrelin alters anxiety- and depression-like behaviour in rats as well as some physiological correlates. Rats bearing chronically implanted ICV catheters were treated with ghrelin (10 μg/day) or vehicle for 4 weeks. Tests used to assess anxiety- and depression-like behaviour were undertaken during weeks 3-4 of the infusion. These revealed an increase in anxiety- and depression-like behaviour in the ghrelin-treated rats relative to controls. At the end of the 4 week infusion, brains were removed and the amygdala dissected for subsequent qPCR analysis that revealed changes in expression of a number of genes representing key systems implicated in these behavioural changes. Finally, given the key role of the dorsal raphe serotonin system in emotional reactivity, we examined the electrophysiological response of dorsal raphe neurons after a ghrelin challenge, and found mainly inhibitory responses in this region. We demonstrate that the central ghrelin signalling system is involved in emotional reactivity in rats, eliciting pro-anxiety and pro-depression effects and have begun to explore novel target systems for ghrelin that may be of importance for these effects.

Keywords: emotional reactivity, anxiety, depression, memory, GHS-R1A, serotonin

Abbreviations

CNS = central nervous system

CV = coefficient of variation

GHS-R1A = growth hormone secretagogue receptor 1A

HPA = hypothalamo-pituitary-adenal

IGF-1 = insulin-like growth factor 1

ICV = intracerebroventricular

I.P. = intraperitoneal

qPCR = quantitative polymerase chain reaction

Cnr1 = cannabinoid receptor 1

Crhr1= corticotrophin releasing hormone receptor 1

Gabra3 = gamma-aminobutyric acid A receptor, alpha 3

Gabra5 = gamma-aminobutyric acid A receptor, alpha 5

Grm5 = glutamate receptor metabotropic 5

Htr1a = serotonin receptor 1a

Syp = synaptophysin

Slc6a3 = solute carrier family 6 (neurotransmitter transporter, dopamine), member 3

1.

1.1. Ghrelin, a stomach-derived hormone (Kojima et al., 1999), increases food intake and fat mass (Tschöp et al., 2000, Theander-Carrillo et al., 2006). Apart from the reported metabolic effects that involve hypothalamic actions it is becoming increasingly apparent that ghrelin's neurobiological actions extend to systems involved in memory (Diano et al., 2006), reward (Jerlhag et al., 2006, Jerlhag et al., 2007, Wellman et al., 2008, Jerlhag et al., 2009) and even to systems involved in the cognitive processing of visual food cues (Malik, 2008). These effects are consistent with the reported distribution of the growth hormone secretagogue receptor (GHS-R1A), the only identified receptor for ghrelin that is present in, for example, discrete hypothalamic, mesolimbic, tegmental and hippocampal areas (Guan et al., 1997, Zigman et al., 2006). Our discovery that ghrelin targets a mesolimbic circuit, the so-called, "cholinergic dopaminergic reward link" (Jerlhag et al., 2006, Jerlhag et al., 2007), a pathway important for the incentive value of natural and artificial rewards, implicates the central ghrelin signalling system in reward-seeking behaviour.

1.2. Given the emerging neurobiology of ghrelin action, surprisingly little is known about its effects on mood. In rodents, acute peripheral, as well as central ghrelin injection (both ICV and site specific: amygdala, dorsal raphe nucleus, hippocampus) induce anxiety-like behaviour (Asakawa et al., 2001, Carlini et al., 2002, Carlini et al., 2004). Furthermore, suppression of central ghrelin action by administration of antisense DNA for ghrelin caused a decrease in anxiety- and depression-like behaviour in rats (Kanehisa et al., 2006). In contrast to these findings, Lutter and colleagues reported a decrease in anxiety- and depression-like behaviour in mice after peripheral ghrelin injection as well as after starvation (Lutter et al., 2008). Surprisingly little is known about the effects of chronic ghrelin exposure on these behaviours, forming a key aim of the present study. Even though, genetic and pharmacological studies suggest that ghrelin may be of clinical relevance for psychiatric

conditions like mood disorders in man (Schmid et al., 2006, Kurt et al., 2007, Nakashima et al., 2008) some contradictory data have been published (Emul et al., 2007, Kluge et al., 2009).

1.3. Here we provide the first description of the effects of chronic stimulation of the central ghrelin signalling system on mood. Thus, in behavioural studies we investigated anxiety- and depression-like behaviour in rats centrally infused with ghrelin for 28 days, and have begun to explore the neurobiological mechanisms underpinning these effects of ghrelin.

2. Experimental procedures

2.1 Animals

Rats were maintained in a controlled environment (12-h light schedule, 21-22°C) with food and water available *ad libitum*. For behavioural/molecular studies, male Sprague–Dawley rats (270–300 g; B&K Universal, Sollentuna, Sweden) were used. After surgery, they were housed individually. Behavioural tests were carried out during the light phase (0800h–1300h). Rats were tested in a balanced order and all behavioural analyses were done by an experimenter who was unaware of the drug treatment. The behaviour was recorded via a camera directly linked to a computer located in the adjacent room. For *in vitro* electrophysiological studies male Sprague-Dawley rats (80-150 g; B&K Universal, Sollentuna, Sweden and Charles River, Germany) were used. All procedures were approved by the Göteborg Animal Experiment Ethics Committee.

2.2 Surgical procedure for central ghrelin administration

Rats were anesthetized (60-75 mg/kg Ketalar® and 0.5 mg/kg Domitor® IP; Pfizer, Sweden; Orion Co, Finland) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). An ICV cannula (Alzet Brain Infusion Kit II) was inserted into the lateral

ventricle using the following coordinates from bregma: AP −0.6, ML -1.4, V: −2.3. The cannula was anchored to the skull with two jeweller screws (EICOM, Japan) and Paladur dental cement (Heraeus Kulzer, City, Germany). The cannula was connected via polyethylene catheter to an osmotic minipump (Alzet Mini-Osmotic Pump Model 2004, Durect, Cupertino, flow rate, 0.25 µl/h for 28 days) implanted subcutaneously in the back of the animals. The catheter and the osmotic pump were filled with saline (NaCl 0.9%) or with saline containing ghrelin (10 µg/animal/day; gift from Rose Pharma A/S, Copenhagen, Denmark). The selected ICV dose of ghrelin was based on previous publications that reported effects on peripheral metabolic parameters (Theander-Carrillo et al., 2006). After surgery, rats were allowed a 15-day recovery period before commencing the behavioural testing.

2.3. Metabolic parameters and hormone measurement

Regular measurements of food intake and body weight were made throughout the study period, and were limited to every 4th day during the behavioural testing period. At the end of the 28 days of treatment, rats were killed by decapitation and fat tissues (mesenteric, retroperitoneal, inguinal, reproductive adipose tissue and brown adipose tissue) were dissected and weighed. Trunk blood was collected in order to measure plasma IGF-1 and corticosterone, hormones that could provide a link between ghrelin action and CNS effects on emotional reactivity. IGF-1 was measured using a radioimmunoassay kit from Mediagnost (Reutlingen, Germany; assay sensitivity was 0.02 nmol/l). Corticosterone levels were determined in 50 µl plasma samples using a radioimmunoassay kit from MP Biomedicals (Orangeburg, NY, USA).

2.4. Behavioural testing

2.4.1. Anxiety-related behaviour was investigated in four widely used tests in rodents: the open field test, the black and white box, the social interaction test and the elevated plus maze (Ramos and Mormede, 1998, File and Seth, 2003, Cryan and Holmes, 2005). Depression-like behaviour was assessed in the forced swim test (Porsolt et al., 1977). The order of the tests was chosen to reduce as much as possible the influence of the successive testing on the outcomes of each test. Thus behavioural tests were arranged such that there was a progressive increase in the stress evoked by the test. A timeline of the experimental schedule is given in figure 1.

2.4.2. Open field test

The open field consisted of a wood box (60 cm x 60 cm x 60 cm) in which the open field was divided into a 25 cm x 25 cm central zone and a border zone surrounding it. The illumination at floor level was 300 lux. In order to evaluate locomotor activity, the floor was divided in 16 equal squares. At the beginning of the test, rats were individually placed in the open field, and their behaviour was analysed during the 10-min test period: time spent in the central zone, the number of entries into the central zone, the number of groomings, the time spent grooming, the number of rearings in the central and peripheral zone and the number of line crossings. The arena was cleaned between each test session.

2.4.3. Black and white box

The box was made of wood and divided into two compartments, connected by an opening (5 x 5 cm wide). The first compartment (18 x 27 x 27 cm high) was painted in black and covered by a black top giving an illumination of 0 lux. The other compartment (27 x 27 x 27 cm high) was painted in white and lit by a white incandescent bulb (100 lux). The floor of the box was cleaned before each trial. At the beginning of each 10-min trial, the rat was

placed in the centre of the white compartment facing the opening. The behavioural parameters scored were: the latency until the first entry into the black compartment and the number of entries into it, the latency until the first entry into the white compartment (after the first entry into the black compartment) and the time spent there as well as the number of rearing into the white compartment.

2.4.4. Elevated-Plus Maze

Two open arms (50 x 10 cm) surrounded by a 1 cm high Plexiglas and two closed arms (50 x 10 x 38 cm high walls) emerged from a central platform. The apparatus was made from dark grey PVC and the arms were elevated 73 cm above the floor. A white incandescent bulb provided a light intensity over the open arms of 100 lux and of 60 lux over the closed arms. The behavioural parameters scored were: the number of entries into the closed arms and in all arms, the number of open arm entries (expressed as percentage of the total number of entries; an entry was counted when both forepaws were placed on the respective arm), the time spent there (expressed as the percentage of the total time spent in all arms), the total time spent on all arms, the number of partial entries, the number of stretched attends in closed and open arms, the number of head dips.

2.4.5. Social interaction test

Two rats from the same treatment group were placed together in the experimental cage in a wood box that was used previously for the open field test under bright light (700 lux) for a 10-min observation period. Interaction time was recorded manually and consisted of active behaviours such as grooming, chasing and playing. The number of entries into the central zone and the time spent there, the number of rearing, the number of line crossings, the latency

until the first self-grooming, the number of grooming and the time spent grooming were analysed. Five rat pairs were tested per treatment group.

2.4.6. Forced-swim test

The procedure was a modification of that described by Porsolt et al. in 1977 (Porsolt et al., 1977). Animals were placed in individual glass cylinder (diameter 17 cm, height 40 cm) containing water (height 24 cm, 22°C). Two swimming sessions were conducted (an initial 15-min pre-test followed the next day by a 6-min test). The duration of immobility, struggling and swimming (in seconds) were measured manually during the 15 min and also during the 6-min of both pre-test and test sessions by an experimenter who was unaware of the drug treatments. Immobility was defined as the minimal movement necessary for the rat to stay afloat. Floating occurred when rats remained immobile with only occasional slight movements to keep the body balance and the nose above the water. Swimming occurred when rats moved all four limbs, swimming around in the tank or diving. Struggling was recorded when rats moved strongly all four limbs with the front paws breaking the water surface or scratching the glass cylinder wall.

2.5. Regulation of candidate genes in amygdala by ghrelin

2.5.1 RNA preparation and cDNA synthesis

Ghrelin- and saline-treated rats were killed by decapitation after 28 days infusion. Brains were rapidly removed, the amygdala was dissected, quickly frozen in liquid nitrogen and stored at -80°C until further processing. Total RNA was purified from individual amygdala samples using RNeasy$^{@}$ Lipid tissue Mini Kit (Qiagen, Hilden, Germany) with additional DNAse treatment (Qiagen). Isolated RNA was diluted in nuclease free water (Ambion, Applied Biosystems, Sundbyberg, Sweden), and RNA concentrations were determined using

spectrophotometer counting. RNA integrity was assured using the RNA 6000 Nano kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA corresponding to 1 μg was reversed transcribed in a total volume of 20 μl, using random hexamers (Applied Biosystems, Sundbyberg, Sweden) and Superscript III reverse transcriptase (Invitrogen Life Technologies, Paisley, UK), according to the manufacturer's description. Recombinant RNaseout® Ribonuclease Inhibitor (Invitrogen) was added to prevent RNase-mediated degradation. All the cDNA-reactions were run in triplicate and the triplicates were pooled together.

2.5.2. Real-time RT PCR using TaqMan® Array

Real-time RT PCR was performed using TaqMan® Custom Array platforms. They were designed with TaqMan probe and primer sets for target genes involved in emotional reactivity, chosen from an on-line catalogue (Applied Biosystems). The sets were factory-loaded into the 384 wells of TaqMan® Arrays. Each port on the TaqMan® Array platforms was loaded with 2 μl cDNA (corresponding to 100 ng total RNA, see above), combined with 48 μl nuclease free water and 50 μl TaqMan® Gene Expression Master Mix (Applied Biosystems) to a final volume of 100 μl. Duplicates of cDNA were run on separate array cards and were analyzed using the 7900HT system with a TaqMan Array Upgrade (Applied Biosystems). Thermal cycling conditions were: 50°C for 2 min, 94.5°C for 10 min, 97°C for 30 s, and 59.7°C for 1 min. To calculate the expression stability of five reference genes (ribosomal protein L27, 18S ribosomal RNA, β-actin, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and Cyclophilin A), the NormFinder algorithm (http://www.mdl.dk/publicationsnormfinder.htm) was used. Based on the intra- and inter-group variability Normfinder calculates the most stable combination of two of the reference genes and this combination is used as reference genes for all the target genes. In our study, a

combination of B-actin and Gapdh were used as reference genes. Gene expression values were calculated based on the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), where the saline-treated group was designated the calibrator. Briefly, ΔC_t represents the threshold cycle (C_t) of the target gene minus that of the reference gene and $\Delta\Delta C_t$ represents the ΔC_t of the ghrelin treated group minus that of the calibrator for each target gene. Relative quantities were determined using the equation; relative quantity = $2^{-\Delta\Delta Ct}$. For the calibrator sample, the equation is relative quantity = 2^{-0}, which is 1; therefore, the expression of the ghrelin-treated group is expressed relative to this.

2.6. Electrophysiological in vitro recording of dorsal raphe neurons

Extracellular single unit recordings were made from cells in a trimmed brain slice preparation (thickness 400 μm) containing the dorsal raphe nucleus. Slices were continuously perfused in a temperature-controlled (35°C) chamber with oxygenated (95% O_2 : 5% CO_2) artificial cerebrospinal fluid containing 5 μM phenylephrine, an α_1-adrenergic receptor agonist (that replaces an endogenous excitatory drive and enhances detection of spontaneously active cells in this region) (Vandermaelen and Aghajanian, 1983). Glass electrodes (10-30 MΩ resistance) were filled 2% pontamine sky blue dissolved in 0.5 M sodium acetate. For each recorded cell, a steady firing pattern was established for at least 10 min before administering 2 μM human ghrelin (gift from Rose Pharma A/B, Copehagen, Denmark) to the slice chamber for 10 min. This dose was selected based on previous electrophysiological studies of ghrelin-responsive neurones in other brain areas (Bajic et al., 2004, Abizaid et al., 2006). Only one cell was tested in each slice. For each cell, a comparison was made of the mean firing rate (Hz) during the 400 s before the application of ghrelin (40x10 s intervals) with the firing rate during the period 200-600 s after the ghrelin had reached the recording chamber. A two sample paired t-test was used to determine whether the firing rate was significantly changed at

P<0.001. Data were expressed as mean ± SEM Hz. As the recordings were extracellular, it was not possible to neurochemically identify the recorded cells by subsequent immunohistochemical labelling. However, previous studies have reported that serotonergic cells in this region tend to have a regular firing pattern at a slow frequency, 0.1 to 3 Hz) (Vandermaelen and Aghajanian, 1983, Hajos and Sharp, 1996). For this reason, we measured the coefficient of variation (standard deviation/mean) of the interspike interval measured 400 s just before the application of ghrelin, to describe the firing pattern.

2.7. Statistical Analyses

2.7.1. Individual behavioural tests

Data obtained in the different tests were analysed either by a Student t-test or by a Mann-Whitney U test when data were not normally distributed or of unequal variance. The results of the forced swim test were subjected to two-way analysis of variance (ANOVA, time x/rat treatment) followed by a Scheffe post hoc test for comparisons between groups when appropriate. In addition, in each case, P<0.05 was considered statistically significant.

2.7.2. Correlation analyses for the behavioural tests

A Pearson correlation was done in order: 1) to study a potential relation between anxiety related parameters (Black and white box: latency until the first entry in the white compartment and the time spent there, the number of transitions, Elevated plus maze: % of time spent and number of entries into the open arms, Open field: number of central entries, number of time spent in the central area, the latency grooming, the number of grooming and the time spent grooming) and locomotor activity related parameters (Open field: number of lines crossing, Elevated plus maze: number of closed and total arm entries); 2) to study a

potential relation between animal body weight and the parameters scored in the forced swim test.

2.7.3. Gene expression analyses

In order to analyse the effect of chronic central ghrelin treatment (versus saline vehicle) on gene expression, Student's t-test was used. P-values and SEM were calculated using the ΔC_t- values. Correction for multiple inference was performed by a permutation step down method (Westfall and Young, 1993) implemented in the R routine comp.adjp. A partial correlation analysis that controlled for treatment was used to explore correlations between the individual genes that showed a significant change in the Student's t-test.

3. Results

3.1. Behavioural tests of anxiety-related behaviour

3.1.1. In the open field test (Figure 2), ghrelin-treated rats displayed a lower number of entries into the central area ($P<0.05$), a decrease in the amount of time spent there ($P<0.01$) and a reduction in the number of central rearings ($P<0.05$). Ghrelin-treated rats spent more time grooming and displayed an increased number of groomings than their saline-treated counterparts ($P<0.05$, $P<0.01$). In the black and white box, ghrelin-treated rats spent less time in the white compartment ($P<0.05$) and tended to display a reduced number of rearings ($P=0.088$, versus controls) (Table 1). In the elevated plus maze (Figure 3), ghrelin-treated rats spent less time on the central platform ($P<0.05$) and there was a tendency to a reduction for the time spent in the open arm ($P=0.057$). The ghrelin-treated group also entered the open arm less often and displayed a lower number of total arm entries (both $P < 0.05$). In the social interaction test the ghrelin-treated rats spent less time in social interaction than the saline-treated rats (72.1 ± 5.7 s versus 125.3 ± 17.1 s $P<0.05$).

3.1.2. As movement inhibition is considered to be characteristic of anxious rats (Escorihuela et al., 1999, Henderson et al., 2004) the observations from the open-field test and the black and white box experiments might be interpreted as anxiety-related behaviour. However, the effect of ghrelin on anxiety does not appear to be contaminated by motor impairment since ghrelin had no effect on the number of line crossings or the number of entries into the closed arm, parameters classically related to locomotor activity in the open field (Prut and Belzung, 2003) and the elevated plus maze (Cruz et al., 1994).

3.2. Behavioural tests of depression-related behaviour

During the first day of the forced swim test, the total time spent in immobility was higher in ghrelin-treated than in saline-treated rats ($P<0.01$, Figure 4A) whereas the total time spent struggling and the total time spent swimming were lower in the ghrelin-treated group

(P<0.001 and P<0.05 respectively, Figure 4A). ANOVA revealed that independently of the day, ghrelin-treated rats spent more time in immobility (P<0.05, Figure 4B) and tended to spend less time struggling and swimming (P=0.058 and P=0.066 respectively, Figure 4B), indicative of increased depression-like behaviour in the ghrelin-treated group. In addition, independently of the treatment, the time spent in immobility was higher the second day than the first day (P<0.01, Figure 4B) and the time spent struggling and swimming were lower the second day (P<0.005 and P<0.05 respectively, Figure 4B). Using a Student t-test to analyse the 6 min behaviour each day, it appears that on the first day, ghrelin-treated rats spent more time in immobility and less time in struggling than the saline-treated rats (P<0.05 and P<0.01 respectively, Figure 4B), whereas on the second day, ghrelin-treated rats spent more time in immobility and less time swimming than the saline-treated rats (P<0.05, Figure 4B).

3.3. Correlation analyses

The Pearson correlation coefficients between the “anxiety parameters” and the “locomotor parameters" selected are significant at P<0.05 (Table 2). Globally, anxiety and locomotor parameters appear correlated only in ghrelin-treated rats. No significant correlation was found between the forced swim test parameters and the locomotor parameters. Pearson correlation coefficients were also calculated between the animal body weight and parameters scored in the forced swim test (Table 3). No correlation between body weight and the latter parameters was found in either the saline-treated or the ghrelin-treated group.

3.4. Molecular analysis of genes associated with emotion

A list of genes investigated in the amygdala, together with relative changes in expression are given in Table 4. The following genes had an increased expression in the ghrelin-treated group: Cnr1, Crhr1, Gabra3, Gabra5, Grm5, Htr1a, Syp and one gene, Slc6a3

had a decreased expression in the ghrelin-treated group compared to the saline-treated. Partial correlation analysis controlling for treatment revealed a significant correlation between Crhr1, Gabra3, Gabra5, Htr1a and Syp ($P<0.05$), indicating that these changes are correlated. When adjusted for multiple comparisons, 2 of the 8 genes (Crhr1 and Syp) remained significant.

3.5. Metabolic parameters and hormonal measurements

A limited number of parameters reflecting ghrelin's orexigenic and adipogenic properties were followed in this 28 day central infusion protocol, largely to confirm biological effects of ghrelin. Body weight gain during the 28 day ICV treatment period was greater for the ghrelin-treated group (163.1 ± 6.9g) than for the saline-treated group (104.5 ± 7.3g: $P<0.001$) as expected (Tschöp et al., 2000, Theander-Carrillo et al., 2006). This was accompanied by a small increase in cumulative food intake in the ghrelin-treated group (833.7 ± 14.5g *versus* 695.3 ± 17.1g: $P<0.001$). The weight of all dissected fat pads (both absolute weight and when expressed per gram body weight) was higher in the ghrelin-treated group than the saline-treated group (33.5 ± 1.9g *versus* 19.1 ± 1.7g: $P< 0.001$). Finally, plasma IGF-1 levels were higher in ghrelin-treated animals than in saline-treated animals ($P<0.05$) but no significant difference was observed for corticosterone levels ($P= 0.78$).

3.6. Electrophysiological in vitro recording of dorsal raphe neurons

Recordings were made from 18 cells in the medial parts of dorsal raphe nucleus in the presence of 5 μM phenylephrine. Phenylephrine was used to replace the stimulation of α_1 receptors, a major excitatory input to dorsal raphe cells in vivo, which is severed in the in vitro slice preparation (Vandermaelen and Aghajanian, 1983). The average firing rate under control conditions was 1.4 ± 0.35 Hz which is in the same range as reported previously (Vandermaelen and Aghajanian, 1983). Half the population of the tested cells (9/18) was

inhibited after bath application of ghrelin (2 μM) from 1.9 ± 0.7 Hz to 1.4 ± 0.6 Hz (Figure 5A) and the coefficient of variation (CV) was 0.3 ± 0.1 under control conditions. The remaining nine cells (9/18) were not affected by bath application of ghrelin (Figure 5B): 0.9 ± 0.12 Hz to 1.0 ± 0.13 Hz (CV = 0.5 ± 0.2, one phasic firing cell excluded; CV=12.9).

4. Discussion

4.1. Here we provide the first demonstration that chronic central ghrelin treatment to rats causes an altered emotional response that appears to reflect an increase in anxiety- and depression-like behaviour. These findings are in line with the reported anxiogenic effects of acute ghrelin injection to rats when administered centrally, peripherally or into discrete brain areas (hippocampus, amygdala and dorsal raphe nucleus)(Asakawa et al., 2001, Carlini et al., 2002, Carlini et al., 2004) and also with studies showing antidepressant and anxiolytic effects after three days administration of antisense DNA for ghrelin to rats (Kanehisa et al., 2006). However, our study contrasts with the conclusion of a study by Lutter and colleagues in which an increase in ghrelin levels by food restriction as well as a subcutaneous injection of ghrelin in mice were shown to cause an anxiolytic- and antidepressant-like behaviour in the elevated-plus-maze and in the forced swim test (Lutter et al., 2008). This discrepancy may be due to different animal species used, differences in route and chronicity of administration. It is also of note that using a chronically food restricted mouse model and peripheral injection (i.p.) of ghrelin, Carlini et al. and Asakawa et al., showed, respectively, an increase of anxiety-related behaviour in the elevated-plus-maze, data also in contradiction with that of Lutter and colleagues (Asakawa et al., 2001, Carlini et al., 2008). We cannot rule out the possibility of GHS-R1A-independent effects, for example, via additional receptors or receptor subtypes for acylated ghrelin; additional receptor subtypes for ghrelin signalling have been suggested (Schellekens et al., 2010) and may have an impact on behaviour (Jiang et al., 2006).

Alternatively, the observed behavioural effects could be mediated by desacyl ghrelin although, to our knowledge, there have not yet been studies investigating relevant behavioural effects of this peptide.

4.2. In humans, only few studies have investigated the relationship between ghrelin and psychiatric conditions like mood disorders. Nevertheless, the results are inconsistent. While some publications found no difference in serum ghrelin in patients with major depressive disorders (Emul et al., 2007, Kluge et al., 2009), there is one report indicating higher serum ghrelin both in patients with major depressive episodes and in those with bipolar disorder-manic episodes (Kurt et al., 2007). Furthermore, some authors suggest that psychopathological improvement of major depression is associated with a significant decrease in serum ghrelin (Schmid et al., 2006, Emul et al., 2007) whereas data obtained with sleep deprivation suggest that the beneficial effects of chronotherapeutic interventions in depression may be mediated by an increase of ghrelin (Schussler et al., 2006, Treuer, 2007). However even though the data concerning the relationship between ghrelin and psychiatric disorders appears conflicting, taken together, these studies in humans and rodents suggest that both short and long term stimulation of the central ghrelin signalling system is likely to be involved in the regulation of mood. This appears in line with the evidence that several peptides involved in the regulation of appetite and metabolism such as the neuropetide Y and the cholecystokinin as well as other hormones like leptin played also a key role in the regulation of emotional process (Lu et al., 2006, Lu, 2007, Liu et al., 2010, Rotzinger et al., 2010).

4.3. In the open field test, chronic central ghrelin treatment increased self-grooming, a behaviour often associated with increased activity of the HPA axis (Steimer and Driscoll, 2003, Frank et al., 2006) and has been reported to be increased in stressed (Moody et al., 1988) as well as in anxious animals (Steimer et al., 1997, Escorihuela et al., 1999). Anxiolytic drugs have been found to suppress novelty-induced self-grooming (Moody et al., 1988). As

reported previously (Theander-Carrillo et al., 2006), central ghrelin infusion did not increase basal plasma levels of corticosterone; this contrasts with data for repeated central injection (Stevanovic et al., 2007) or i.p. injection (Asakawa et al., 2001). In this context, ghrelin administration has been shown to facilitate ACTH release via activation of CRH and vasopressin release (Korbonits et al., 2004). In our study, since corticosterone concentrations have been measured in the basal situation but not after the stress, we cannot rule out the possibility that the HPA axis might have been enhanced in ghrelin-treated rats and thus increase the self-grooming behaviour observed. Interestingly, in the gene expression studies we found an increased expression of corticotrophin releasing hormone receptor 1 (Crhr1) mRNA in the amygdala of ghrelin-treated rats, an effect associated with increased anxiety-like behaviour in other contexts (Liebsch et al., 1995). Induction of this receptor in the amygdala by ghrelin may indicate that at least this component of the central HPA axis remains a target for ghrelin in the chronic treatment model.

4.4. In man, there appears to be a strong association between disorders of anxiety and those of depression evidenced, for example, from epidemiological studies of their comorbidity (Kessler et al., 1999, de Graaf et al., 2002). In the present study, therefore, we also assessed depression-like behaviour using the forced swim test. This test continues to be used extensively for evaluating antidepressant drugs and the behavioural pattern of anxiety/depression in rats (Porsolt et al., 1977, Cryan and Holmes, 2005, Hinojosa et al., 2006). In the forced swim test, the ghrelin-treated rats showed an increase in immobility and a decrease in active movements indicative of an increase in depression-like behaviour. This is also in agreement with the decreased immobility in the forced swim test after treatment with antisense DNA for ghrelin (Kanehisa et al., 2006).

4.5 In the forced swim test, it is important to consider whether ghrelin-related changes in behaviour, that we interpret to reflect altered emotional reactivity, could be explained by

independent effects such as motor disturbance or indeed, changes in body weight and/or muscle mass. Motor disturbance seems unlikely because indicators of locomotor activity from the other behavioural tests, were unaltered by ghrelin treatment (e.g. closed arm entries in the elevated plus-maze test and number of line crossing in the open field test). In addition, we did not find any significant correlation between these indices and the parameters scored in the forced swim test. Although body weight and lean body mass are increased by 2 weeks central ghrelin treatment (Salome et al., 2009), we did not find any indication that changes in body composition are responsible for the observed effects of ghrelin treatment in the forced swim test. Thus, for example, ghrelin treatment caused a decrease in active movements, which would not be consistent with an increased muscle mass. Also, correlation analysis did not show any relationship between body weight and the parameters measured in the forced swim test. Thus, the ghrelin-induced effects on these parameters are independent of any increase in body fat or muscle mass.

4.6. There are no good ways of controlling for the ghrelin-induced weight gain in the behavioural tests. Food restricting the ghrelin-treated rats would stress them in a way that would likely affect behavior. A similar argument is true for force feeding of saline-treated rats. Moreover, feeding the saline treated animals a high fat diet would induce an unknown parameter and we would not be able to compare the effect of the treatment between the ghrelin treated and the saline-treated- HFD fed group. Even though obesity induced by high fat diet affects emotional reactivity in rodents, it appears that the negative effects on mood are a question of caloric quality rather than caloric quantity (Abildgaard et al., 2010). Thus, since our study both groups get normal chow, we assume that behavioural differences observed between ghrelin-treated versus control rats is a consequence of ghrelin on brain circuits involved in emotionality.

4.7 In our study, there were twelve days separating the first and last behavioural test. We cannot rule out the possibility, therefore, that the duration of treatment influences the behavioural response. However, since the difference in emotional reactivity between treated versus non-treated rats is consistently in the same direction during the treatment we can assume that effect of treatment starts at least on day twelve and continues through to the last experimental day.

4.8. As an initial exploration of the consequences of increased central ghrelin signalling on emotionality, and the underlying mechanisms, we examined the relative expression levels of a number of key genes that have been associated with an altered emotionality. We focused on the amygdala, a relevant brain region involved in the regulation of emotional response (Carlini et al., 2004, Toth et al., 2009, Toth et al., 2010). In addition to the aforementioned receptor, Crhr1, the data point to a number of interesting novel potential target genes for ghrelin's central effects, although further confirmation and exploration would be required to demonstrate that these are important for ghrelin's effects on mood.

4.9. In relation to anxiety and depression, the amygdala is suggested to have an important role in assessing the significance of fearful stimuli and generation of behavioural responses to stress and anxiety (Sajdyk and Shekhar, 1997, Aroniadou-Anderjaska et al., 2007). The severity of depression has been positively correlated with the cerebral glucose metabolic rate within the amygdala (Abercrombie et al., 1998) and that the abnormal activity could be normalized by antidepressant drug treatment (Drevets et al., 2002). Also a hyper-active and/or hyper-responsive amygdala is usually found in anxiety disorders (Stein et al., 2002). Therefore, the balance between the activity of excitatory and inhibitory amino acids in the amygdala has been suggested to be of importance for setting the level of anxiety (Sajdyk and Shekhar, 1997). In the present study, the ghrelin-treated animals had increased expression of glutamate receptor metabotropic 5 (Grm5) mRNA. Activation of this receptor is important for

the regulation of GABAergic release onto projection pyramidal cells in this area (Braga et al., 2003). Consistent with this, we also observed an increase in both $GABA_A$-3α (Gabra3) and $GABA_A$-5α receptor (Gabra5) subunit expression mRNA in the amygdala of ghrelin-treated rats.

4.10. In the gene expression analysis, there were indications that ghrelin also influences the central serotonin system reflected by, for example, the increased expression of 5-HT_{1A}-receptor (Htr1a) mRNA in the amygdala of the ghrelin-treated rats. This is unlikely to be a direct effect, given the low level of expression of GHS-R1A in the amygdala (Zigman et al., 2006) but may rather reflect, for example, the activation of serotonergic afferents. Consistent with this, ghrelin has been shown to induce an anxiogenic response when administered to the dorsal raphe nucleus (Carlini et al., 2004) (a major source of serotonergic projections) and, in the present study to inhibit the electrical activity of dorsal raphe cells (notably those with a regular firing pattern). Given that serotonergic cells in this region have a more regular firing pattern (Martin-Ruiz and Ugedo, 2001), we may infer that the ghrelin-inhibited cell group likely include serotonergic cells in this region. The idea that inhibition of serotonin neurons in the dorsal raphe may underlie the increase of anxiety- and depression-like behaviour in rodents is also supported by other reports (Temel et al., 2007).

The central endocannabinoid system appears to be important for ghrelin's orexigenic effects (Tucci et al., 2004, Kola et al., 2008) and it will be interesting to discover whether it is also important for ghrelin's effects on mood. In the present study, we found that chronic central ghrelin treatment increased cannabinoid receptor 1 (Cnr1) expression in the amygdala. Tentatively, by increasing Cnr1 signalling in the amygdala, ghrelin would be expected to reduce serotonin release in this region (Ashton et al., 2006).

4.11. Another interesting candidate gene in the amygdala that was regulated by ghrelin

includes synaptophysin (Syp), that also showed increased expression in ghrelin-treated rats. Interestingly, this gene has previously been associated with fear conditioning (Nithianantharajah and Murphy, 2008). We also observed a decreased mRNA expression of the dopamine transporter (Slc6a3) in the amygdala, a potentially interesting finding given that depressed human subjects have a decreased expression of dopamine transporters in this region (Klimek et al., 2002) and also because reduced dopaminergic activity in the amygdala has anxiolytic effects in rats (de la Mora et al., 2005).

5. Conclusion

From these data we conclude that chronic elevated ghrelin levels in the central nervous system increases anxiety- as well as depression-like behaviour in rats. We also detected changes in mRNA expression for a number of genes in the amygdala which have previously been associated with anxiety and depression. Our electrophysiological studies also highlight the dorsal raphe as a potentially important target for ghrelin in this context. The central ghrelin signalling system emerges, therefore, as a key element in the integrated neurobiology of food intake, reward and mood, increasing food intake, inducing food/drug seeking behaviour and eliciting adverse effects on mood. Our finding that chronic stimulation of the central ghrelin signalling system has adverse effects on mood is of therapeutic and clinical relevance for the development of this system, including the known ghrelin receptor GHS-R1A, as a potential target for the regulation of body weight, for eating disorders, for substance abuse and also for mood disorders.

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Figure legends

Figure 1. Timeline of the experimental schedule.

Figure 2. Behavioural parameters for the saline- and ghrelin-treated groups measured in the open field test. Rats were individually placed in the open field during the 10 min test period. Values are expressed as mean ± SEM (* $P<0.05$, ** $P<0.01$). Saline group: n=11, ghrelin group: n=11.

Figure 3. Behavioural parameters for the saline- and ghrelin-treated groups measured during 5 min in the elevated plus maze. Values are expressed as mean ± SEM (* $P<0.05$). Saline group: n=11, ghrelin group: n=11.

Figure 4. Behavioural parameters measured during the 15 minutes of the first day's forced swim test (A). Parameters for the first 6 min of the pre test phase, day one, and during the 6 min of the test phase on day two (B). Values are expressed as mean ± SEM (* $P<0.05$, ** $P< 0.01$). Saline group: n=11; ghrelin group n=11.

Figure 5. Representative recordings of the firing rate from a cell inhibited (A) and a cell not affected (B) by 2 μM ghrelin.

Table legends

Table 1. Behavioural parameters of saline- and ghrelin-treated rats in the black and white box during the 10 min test period. Values are expressed as mean ± SEM (*P<0.05). Saline group: n=11; ghrelin group n=11.

Table 2. The correlation coefficients for the two treatment groups, between the selected "anxiety parameters" and "locomotor parameters". Numbers in bold are significant at P<0.05.

Table 3. Correlation analysis of animal body weight and parameters scored in the forced swim test.

Table 4. Gene expression changes in genes associated with emotional processes. Values are expressed as relative quotient (+/- SEM) of the ghrelin group (n=8) compared to the saline group (n=8). [a] p-value using Student's t-test. [b] p-value corrected for multiple inference using a permutation step down method.

Hansson et al. Table 1

	Saline	Ghrelin
Rearing	17.36 ± 2.35	11.27 ± 2.47
Latency Black	12.45 ± 2.95	9.18 ± 1.85
Latency White	164.64 ± 35.14	247.09 ± 69.31
Transitions Number	9.64 ± 1.00	7.00 ± 1.38
Time White	179.29 ± 21.37	109.19 ± 20.94*

Hansson et al. Table 2.

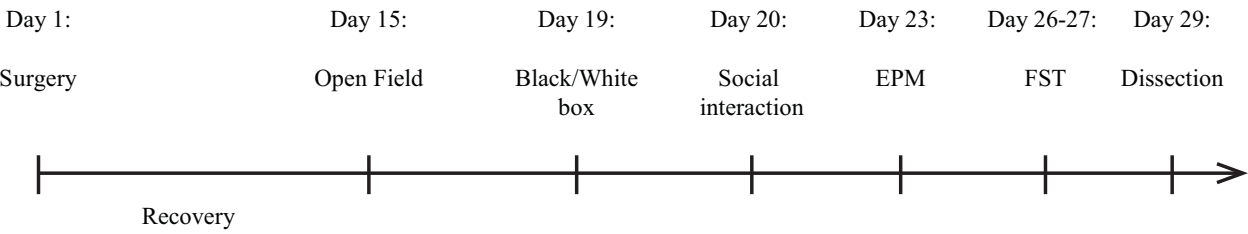
	Variable	Closed arm entries		Total arm entries		Line crossing	
		Saline	Ghrelin	Saline	Ghrelin	Saline	Ghrelin
BWB	Latency white	-0.19	-0.45	-0.69	-0.52	-0.01	**-0.64**
	Number of transitions	0.30	**0.71**	0.54	**0.73**	0.35	**0.85**
	Time white	0.05	0.59	0.57	**0.60**	0.19	**0.65**
EPM	% Time OA	-0.46	0.56	0.25	**0.70**	-0.01	0.27
	% OA Entries	**-0.68**	0.23	-0.03	0.43	-0.22	0.07
OF	Central entries	0.30	**0.68**	0.31	**0.61**	0.30	**0.88**
	Time center	-0.32	**0.73**	-0.17	**0.70**	0.04	**0.93**
	Grooming latency	0.09	0.20	-0.01	0.14	-0.33	0.32
	Time grooming	0.31	**-0.65**	-0.07	-0.57	0.02	**-0.74**
	Number of grooming	0.35	-0.05	0.12	0.06	-0.03	0.09
FST	Total immobility	0.15	-0.03	0.13	-0.03	0.23	0.60
	Total struggling	-0.13	-0.17	0.07	-0.16	-0.17	-0.36
	Total swimming	-0.09	0.14	-0.17	0.22	-0.16	-0.56
	Immobilty first day (6 min)	-0.33	-0.25	0.02	-0.30	-0.20	0.44
	Struggling first day (6 min)	0.07	0.25	0.14	0.26	-0.14	-0.18
	Swimming first day (6min)	0.29	0.23	-0.07	0.28	0.23	-0.46
	Immobility second day	0.05	-0.11	0.24	-0.19	0.46	0.43
	Struggling second day	-0.06	0.26	0.07	0.27	-0.39	-0.15
	Swimming second day	-0.01	0.01	-0.33	0.11	-0.21	-0.48

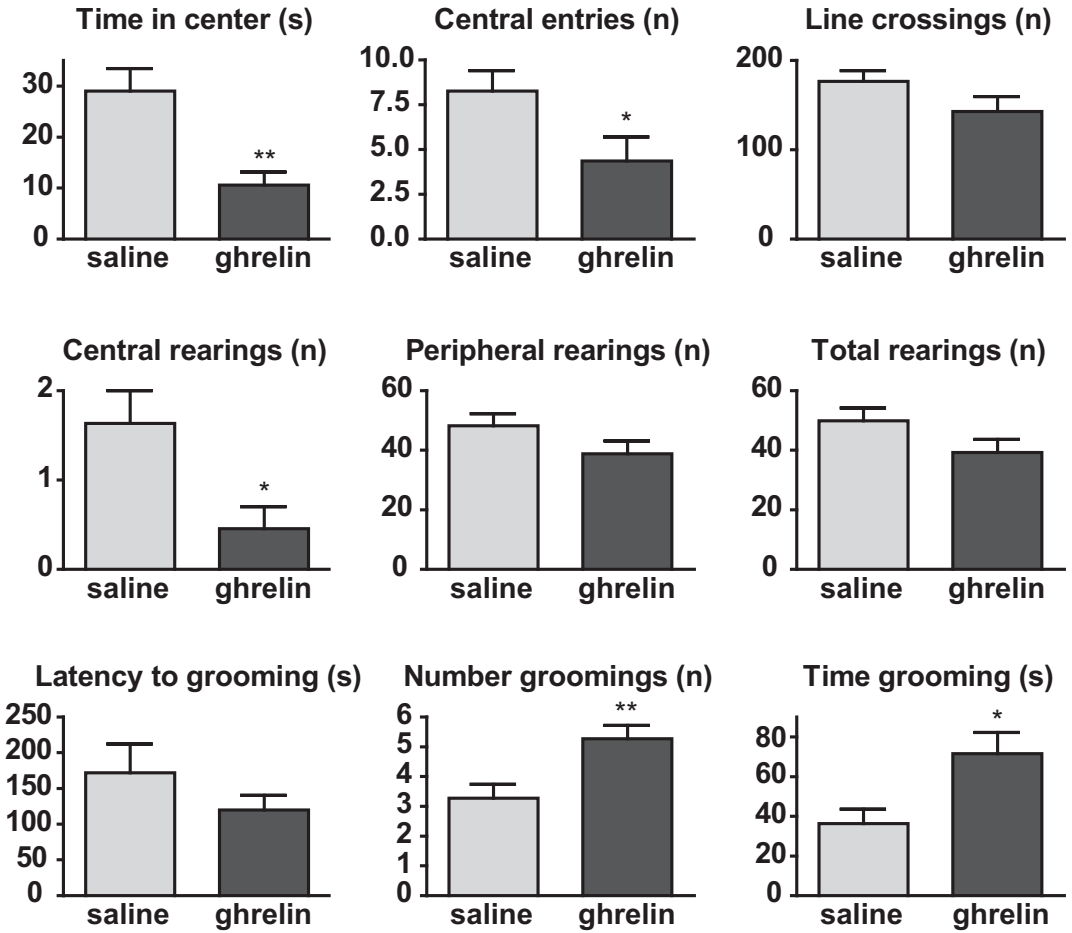
Hansson et al. Table 3.

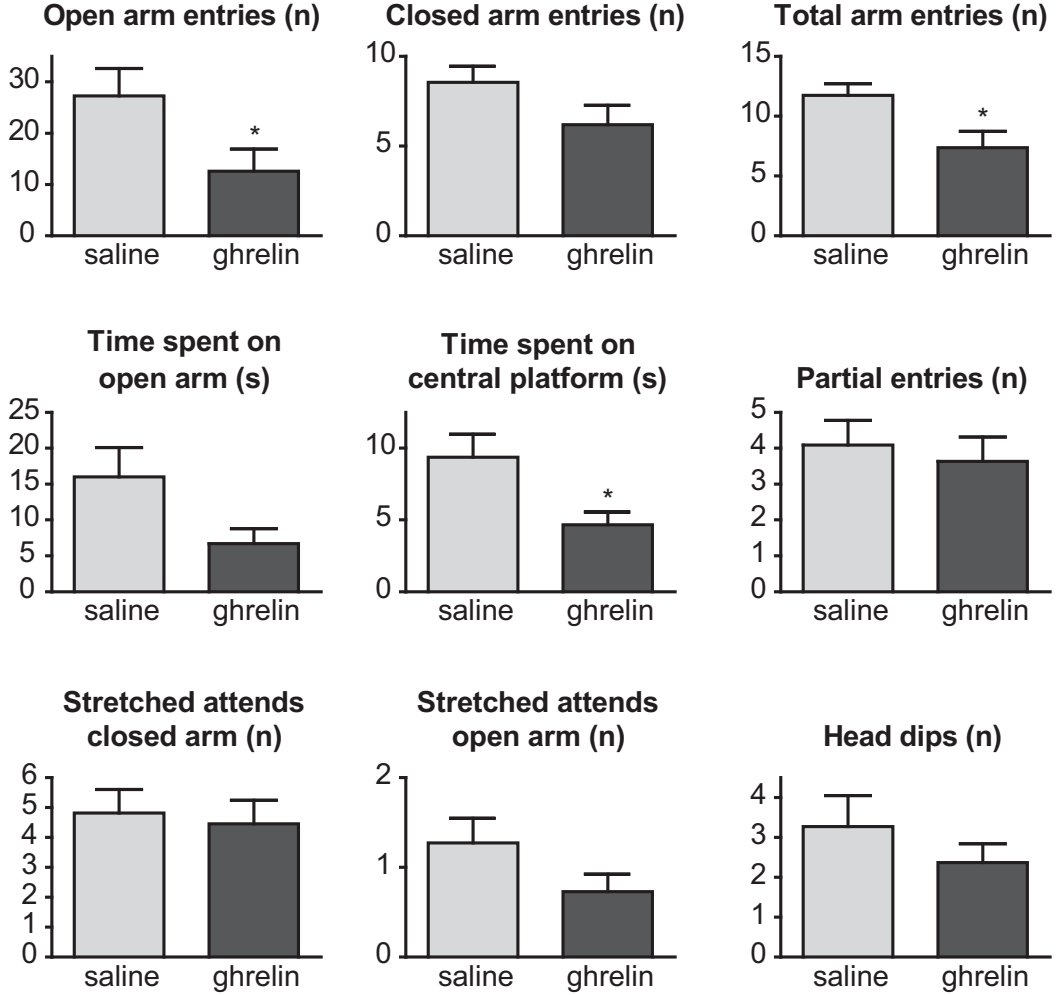
	Weight	
	Saline	Ghrelin
Total immobility	0.09	0.49
Total struggling	-0.00	-0.39
Total swimming	-0.09	-0.47
Immobility first day	0.35	0.56
Struggling first day	-0.13	-0.46
Swimming first day	-0.29	-0.50
Immobility second day	-0.14	0.37
Struggling second day	0.43	-0.25
Swimming second day	-0.07	-0.32

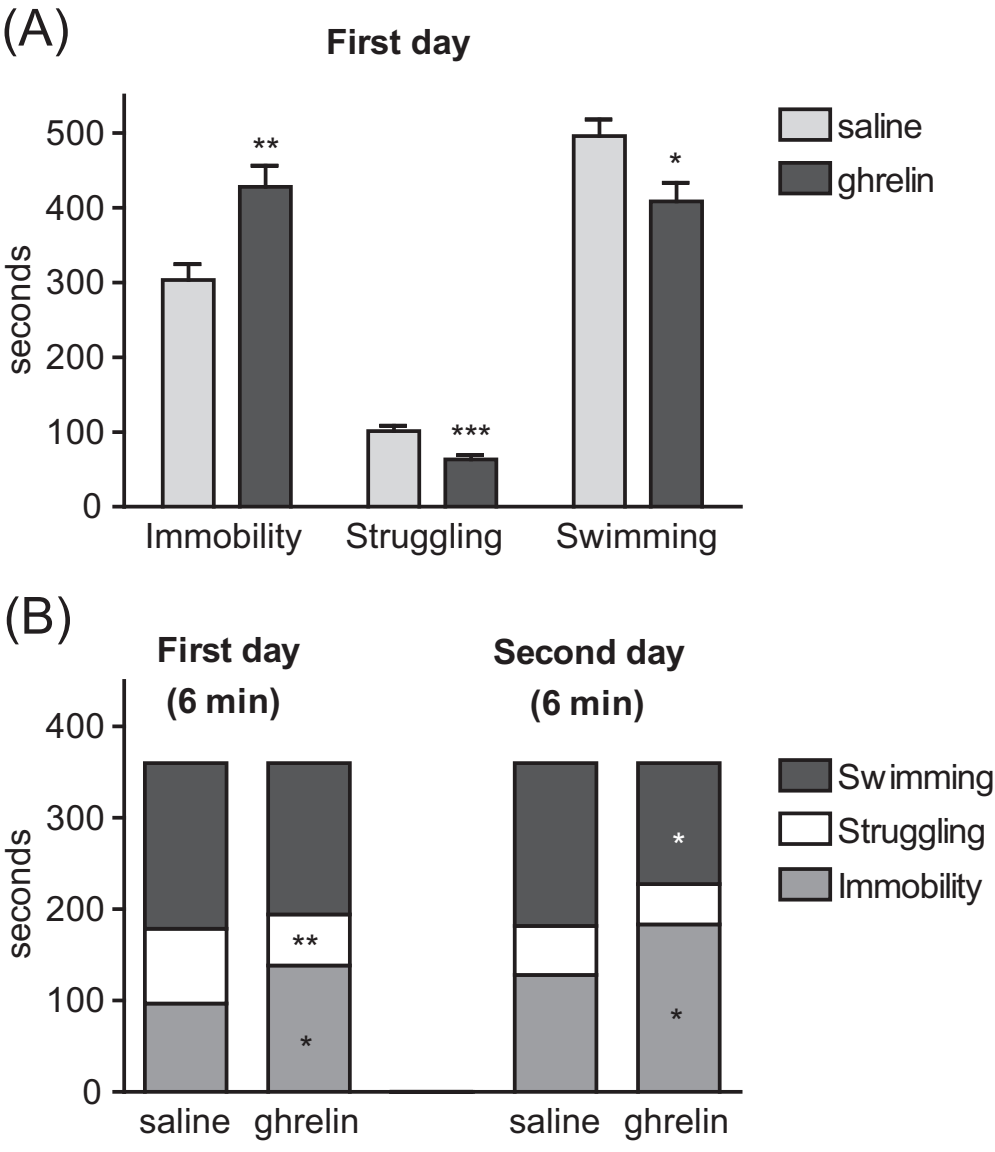
Hansson et al. Table 4.

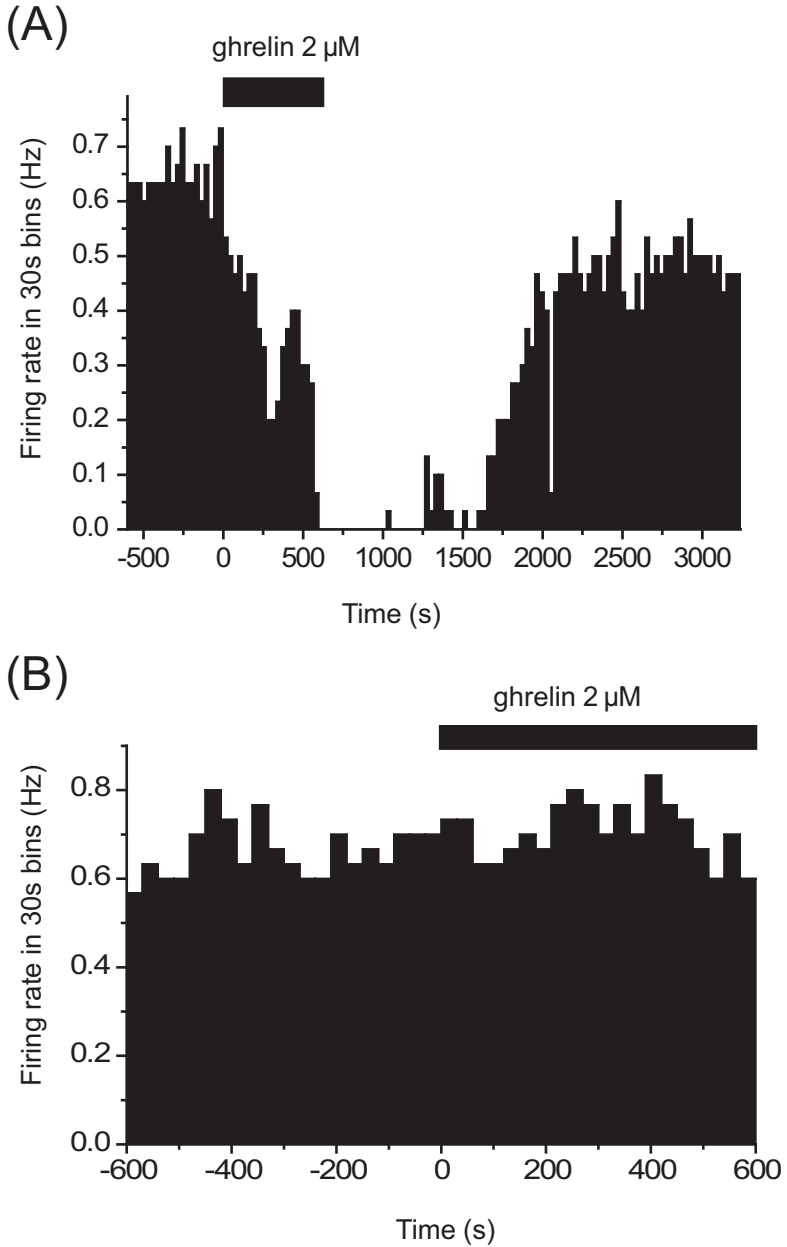
	Saline (+/- SEM)	Ghrelin (+/- SEM)	p-value [a]	adjusted p-value [b]
HPA axis				
Corticotropin releasing hormone receptor 1 (Crhr1)	1 (0.91-1.09)	1.45 (1.38-1.52)	0.003	0.012
Corticotropin releasing hormone receptor 2 (Crhr2)	1 (0.83-1.20)	1.03 (0.95-1.12)	0.875	0.994
Glucocorticoid receptor (Nr3c1)	1 (0.91-1.10)	1.19 (1.16-1.22)	0.116	0.528
Mineralcorticoid receptor (Nr3c2)	1 (0.95-1.05)	1.02 (0.98-1.05)	0.773	0.986
Neurotransmitter systems				
Catechol-O-methyl transferase (Comt)	1 (0.95-1.05)	1.16 (1.08-1.25)	0.109	0.528
gamma-aminobutyric acid A receptor, alpha 1 (Gabra1)	1 (0.95-1.05)	1.11 (1.09-1.14)	0.081	0.510
gamma-aminobutyric acid A receptor, alpha 3 (Gabra3)	1 (0.88-1.14)	1.37 (1.31-1.44)	0.037	0.328
gamma-aminobutyric acid A receptor, alpha 4 (Gabra4)	1 (0.90-1.12)	1.18 (1.14-1.23)	0.173	0.627
gamma-aminobutyric acid A receptor, alpha 5 (Gabra5)	1 (0.90-1.12)	1.29 (1.25-1.34)	0.043	0.345
Glutamate acid decarboxylase 1 (Gad1)	1 (0.85-1.17)	1.01 (0.97-1.05)	0.939	0.994
Glutamate receptor metabotropic 2 (Grm2)	1 (0.80-1.25)	1.18 (1.03-1.35)	0.538	0.969
Glutamate receptor metabotropic 5 (Grm5)	1 (0.86-1.16)	1.41 (1.35-1.48)	0.040	0.328
Serotonin receptor 1a (Htr1a)	1 (0.89-1.12)	1.34 (1.29-1.39)	0.028	0.247
Serotonin receptor 2c (Htr2c)	1 (0.85-1.18)	1.13 (0.99-1.29)	0.570	0.969
Solute carrier family 1 (glial high affinity glutamate transporter), member 2 (Slc1a2)	1 (0.93-1.08)	1.18 (1.14-1.21)	0.060	0.421
Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3 (Slc6a3)	1 (0.77-1.30)	0.33 (0.26-0.42)	0.010	0.079
Synapses / Nerve terminals				
Cannabinoid receptor 1 (Cnr1)	1 (0.93-1.08)	1.2 (1.16-1.24)	0.042	0.345
Mss4 protein (Mss4)	1 (0.93-1.07)	1 (0.97-1.03)	0.982	0.994
Synaptophysin (Syp)	1 (0.94-1.06)	1.23 (1.20-1.26)	0.006	0.044
Synaptotagmin (Syt4)	1 (0.95-1.05)	1.11 (1.08-1.14)	0.081	0.510


Day 1:
Surgery
Day 15:
Open Field
Day 19:
Black/White box
Day 20:
Social interaction
Day 23:
EPM
Day 26-27:
FST
Day 29:
Dissection
Recovery


Time in center (s)
30
20
10
0
**
saline
ghrelin
Central entries (n)
10.0
7.5
5.0
2.5
0.0
*
saline
ghrelin
Line crossings (n)
200
100
0
saline
ghrelin
Central rearings (n)
2
1
0
*
saline
ghrelin
Peripheral rearings (n)
60
40
20
0
saline
ghrelin
Total rearings (n)
60
40
20
0
saline
ghrelin
Latency to grooming (s)
250
200
150
100
50
0
saline
ghrelin
Number groomings (n)
6
5
4
3
2
1
0
**
saline
ghrelin
Time grooming (s)
80
60
40
20
0
*
saline
ghrelin


Open arm entries (n)
30
20
10
0
*
saline
ghrelin
Closed arm entries (n)
10
5
0
saline
ghrelin
Total arm entries (n)
15
10
5
0
*
saline
ghrelin
Time spent on open arm (s)
25
20
15
10
5
0
saline
ghrelin
Time spent on central platform (s)
10
5
0
*
saline
ghrelin
Partial entries (n)
5
4
3
2
1
0
saline
ghrelin
Stretched attends closed arm (n)
6
5
4
3
2
1
0
saline
ghrelin
Stretched attends open arm (n)
2
1
0
saline
ghrelin
Head dips (n)
4
3
2
1
0
saline
ghrelin


(A)
First day
seconds
500
400
300
200
100
0
**
***
*
saline
ghrelin
Immobility
Struggling
Swimming
(B)
First day
(6 min)
Second day
(6 min)
seconds
400
300
200
100
0
**
*
*
*
Swimming
Struggling
Immobility
saline
ghrelin
saline
ghrelin


(A)
ghrelin 2 μM
Firing rate in 30s bins (Hz)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
-500
0
500
1000
1500
2000
2500
3000
Time (s)
(B)
ghrelin 2 μM
Firing rate in 30s bins (Hz)
0.0
0.2
0.4
0.6
0.8
-600
-400
-200
0
200
400
600
Time (s)