TITLE

Acute and chronic suppression of the central ghrelin signaling system reveals a role in food anticipatory activity.

ABBREVIATED TITLE

Central ghrelin antagonism inhibits food-anticipatory activity

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ABSTRACT

Using the rodent activity-based anorexia (ABA) model that mimics clinical features of anorexia nervosa that include food restriction-induced hyperlocomotion, we found that plasma ghrelin levels are highly associated with food-anticipatory behaviour, measured by running wheel activity in rats. Furthermore, we showed that ghrelin receptor (GHS-R1A) knockout mice do not anticipate to food when exposed to the ABA model, unlike their wild type littermate controls. Likewise, food anticipatory activity in the ABA model was suppressed by a GHS-R1A antagonist administered either by acute central (ICV) injection to rats or by chronic peripheral treatment to mice. Interestingly, the GHS-R1A antagonist did not alter food intake in any of these models. Therefore, we hypothesize that suppression of the central ghrelin signaling system via GHS-R1A provides an interesting therapeutic target to treat hyperactivity in patients suffering from anorexia nervosa.

1. INTRODUCTION

Since its discovery in 1999, the peptide ghrelin has emerged as an important gut-brain signal in the control of energy balance (Hosoda et al., 2002;Kojima et al., 1999). The ghrelin receptor (growth hormone secretagogue receptor 1A, GHS-R1A) is highly expressed in the hypothalamus, in particular the arcuate nucleus (Guan et al., 1997). Caloric restriction increases ghrelin secretion, and subsequent activation of the central ghrelin signaling system via GHS-R1A in the arcuate nucleus has been implicated in the stimulation of food intake (Hosoda et al., 2002). In contrast to many endocrine signals, plasma ghrelin levels are elevated prior to meal initiation, decreasing once again during the post-prandial period (Cummings et al., 2001). In addition, acute central or peripheral ghrelin injection stimulates food intake in rats (Horvath et al., 2001;Naleid et al., 2005). Peripheral ghrelin injection also induces appetite in healthy subjects (Wren et al., 2001). Furthermore, ghrelin has been implicated in the response to long term changes in body weight (Cummings, 2006). Collectively, these data are indicative of a physiological role for ghrelin in hunger and meal initiation.

Circulating total ghrelin levels are decreased in obese individuals but can be restored after weight loss (Hansen et al., 2002;Soriano-Guillen et al., 2004). Conversely, total ghrelin levels are elevated in situations of negative energy balance but can be restored by weight regain (Janas-Kozik et al., 2007;Otto et al., 2001;Soriano-Guillen et al., 2004). In patients suffering from anorexia nervosa, total plasma ghrelin concentrations of underweight subjects are increased and tend to normalize with the recovery of body

weight (Otto et al., 2001;Otto et al., 2005). It has been reported by Misra and colleagues that high plasma levels of total ghrelin induced by fasting in humans are negatively associated with the percentage body fat and with low levels of leptin and insulin, which might play an important role in the pathophysiology of anorexia nervosa (Misra et al., 2005).

Activity-based anorexia (ABA) is an animal model of anorexia nervosa, mimicking important characteristics of this disease that include, in particular, increased locomotor activity and reduced food consumption (Routtenberg and Kuznesof, 1967), together with similar endocrine abnormalities (de Rijke et al., 2005). In this model, rodents are given free access to a running wheel and fed once per day for a limited period of time (1-2 h). Exposure to the ABA model leads to a chronic catabolic state caused by a reduced food intake and increased running wheel activity. Increased activity, especially prior to food intake (when ghrelin levels are presumably high), is a hallmark feature of ABA and similar to hyperactivity observed in patients suffering from anorexia nervosa. Interestingly, acute peripheral or central ghrelin administration to *ad libitum* fed mice stimulates locomotor activity (Jerlhag et al., 2006). Conceivably, increased circulating levels of ghrelin may contribute to hyperlocomotor activity.

Recent studies in GHS-R1A KO mice have implicated the central ghrelin signaling system in food anticipatory activity (Blum et al., 2009;LeSauter et al., 2009). Therefore we first investigated whether changes in plasma ghrelin levels were associated with the development of hyperactivity and/or feeding responses in the ABA model. Inspired by

the possibility to use GHS-R1A antagonists to suppress anorexia-induced hyperlocomotor activity, we sought to determine whether food anticipatory activity is suppressed in rodents administered a GHS-R1A antagonist, either by acute central injection or by chronic peripheral exposure. Moreover, we sought to link the hyperlocomotor response more directly to GHS-R1A signaling by eliminating the possibility that caloric restriction provides the primary drive for the food anticipatory response, incorporating studies in both GHS-R1A KO mice and GHS-R1A antagonist treated rats.

2. MATERIAL AND METHODS

Animals

Female outbred Wistar WU rats (n=77) (Harlan, Horst, The Netherlands), weighing 155-165 gram upon arrival, were used to study the correlation between plasma ghrelin levels and hyperactivity and to determine the effect of central GHS-R1A antagonist treatment. Twelve week old female C57Bl/6 mice (n=24) (Harlan, Horst, The Netherlands) were used for studies of peripheral GHS-R1A antagonist treatment. Heterozygous mice for GHS-R1A disruption were originally obtained from Deltagen (San Carlos, CA, USA) and bred in house to generate female GHS-R1A KO and wild type littermate control mice used in the study (Egecioglu et al Addiction Biol 2010). All animals were individually housed in an ambient temperature- and humidity-controlled room (21°C ± 2°C) under a 12-hour dark-light cycle, lights off at 2 pm. The animals were allowed to acclimatize to

the animal facilities for one week prior to any treatment or experiment. All procedures in rats were approved by the ethical committee on the use and care of animals of the University of Utrecht (The Netherlands) and all studies in mice by the local ethics committee for animal experimentation in Gothenburg. For ethical reasons, it was decided that the animals had to be removed from the experiment when their body temperature was lower than 33°C before feeding or when the animals lost more than 25% of their initial body weight.

Measurement of plasma ghrelin in rats exposed to the ABA model.

Rats were individually housed and approximately half of them began training in the ABA model. During a 10 day training period (day -10 to 0), the ABA group (n=29) were given free access to a running wheel and running wheel activity was continuously registered using a Cage Registration Program (Department Biomedical Engineering, UMC Utrecht, The Netherlands). Until the beginning of day 0, the ABA rats were allowed water and food *ad libitum*. At the beginning of the dark phase of day 0, food was removed and animals were placed on the scheduled feeding of 1 hour per day. The control group for the study were not given access to running wheels either prior to or during the study and were pair fed to the ABA group (pair-fed sedentary, n=24). Thus, the experiment was staggered to allow pair-fed feeding: at dark onset pair-fed rats received the average amount of food eaten by the ABA rats the day before. The body weight of all animals was measured just prior the dark phase, and just before food access in both experimental groups. Every consecutive day of the ABA model (ABA day 0-5), 4-5 animals were sacrificed by decapitation at the end of the light phase (see Figure 1). Trunk blood was

collected into lithium-heparin tubes (Sarstedt, Nümbrecht, Germany) containing 83 μmol EDTA and 1 mg aprotonin. Tubes were collected on ice until centrifugation (20 min at 3000 rpm 4˚C). Plasma was stored at -20˚C until assay. Brains were quickly removed, frozen and stored at -80˚C. Plasma levels of total ghrelin (Phoenix Pharmaceuticals, Belmont, CA, USA) were measured by commercially available radioimmunoassay (RIA) kits according to the manufacturer's protocol. From the plasma samples, 2× 50 μl was taken for measurement in duplicate.

Studies exploring the effect of pharmacological suppression of endogenous ghrelin signaling on food anticipatory activity in the ABA model.

In order to make continuous measurements of body temperature and locomotor activity, all rats (n=24) were implanted with transmitters (TA10TA-F40 Data Sciences International, St. Paul, Minnesota) in the abdominal cavity under fentanyl/fluanisone (0.1 ml/100 g body weight, IM; Hypnorm, Janssen Pharmaceutica, Beerse, Belgium) and midazolam (0.05 ml/100 gram body weight, IP; Dormicum, Hoffman-LaRoche, Mijdrecht, The Netherlands) anesthesia. After surgery, rats were supportive treated with the anti-inflammatory agent carprofen (0.01 ml/100 gram body weight, s.c.; Rimadyl, Pfizer Animal Health, Capelle a/d Ijssel, The Netherlands) and saline (3 ml, s.c.) and allowed to recover for at least two weeks. In order to investigate the effects of pharmacological suppression of endogenous ghrelin on activity behaviour and food intake, a GHS-R1A antagonist was administered via a chronically implanted ICV catheter. At day -10, animals were anesthetized (as indicated above) and provided with an ICV cannula placed into the lateral ventricle; coordinates were 1.0 mm posterior from

bregma, 1.0 mm lateral from midline, 5 mm below the surface of the brain, and finally fixed in place with two small screws and dental cement. After recovery, animals were individually housed in cages with running wheels for a training period of ten days (from day -10 to day 0). Running wheel activity was continuously registered. Until the beginning of day 0, animals were allowed food and water *ad libitum.* Animals were divided into experimental groups, matched for body weight and baseline running wheel activity. Baseline running wheel activity was determined as the average running wheel activity during four days prior to the start of scheduled feeding (day -8 to day -4). Body weight of all animals was measured just prior to the one hour period of food access at the beginning of the dark phase (see Figure 1). At the end of day -4 and day 4 of the experiment, just before animals normally display their daily hyperactivity, rats received an ICV injection of a GHS-R1A antagonist (concentration 4, 8 or 16 µg) or an equal volume (3µl) of saline vehicle. The GHS-R1A antagonist was kindly provided by Æterna Zentaris GmbH (ghrelin receptor (GHS-R1A) antagonist, JMV2959, Frankfurt am Main, Germany). At the end of day 5, animals were sacrificed by decapitation as described before.

One day prior to the start of habituation to the running wheel setup female C57Bl/6 mice were anaesthetized with isofluorane and surgically implanted with a subcutaneous osmotic pump (model 1002, 12 day pump from Alzet, DURECT, Cupertino California) delivering either vehicle (saline) or JMV2959 (6 or 12 mg/kg/day) for the duration of the experiment (9 days total). The surgical procedure lasted for approximately 5 min. The following day the animals were individually housed in cages with running wheels for a

training period of 5 days (from day -5 to day 0) with *ad libitum* access to chow and water. On day 0, food was removed 2h into the dark phase and the animals placed on scheduled feeding for 2h each day from the onset of the dark phase. Running wheel activity was monitored continuously, food intake was monitored daily and body weights were recorded before the onset of the dark phase. The animals were killed following 3 days on the scheduled feeding.

Studies exploring the effect of genetic knockout of the ghrelin receptor, GHS-R1A, on food anticipatory activity in the ABA model.

Female GHS-R1A KO and wild type littermate mice were individually housed in cages with running wheels for a training period of 5 days (from day -5 to day 0) with *ad libitum* access to chow and water. On day 0, food was removed 2h into the dark phase and the animals were placed on scheduled feeding for 2h each day from the onset of the dark phase. As differences in anorexic response (fatigue) secondary to changes in caloric intake may itself induce divergent food anticipatory activity responses, and given that young female GHS-R1A KO mice are not able to adapt their food intake on a 4h feeding schedule (Blum et al., 2009) a third experimental group was introduced, namely female GHS-R1A KO mice pair-fed to eat the same amount of food as the wild type mice. In this group, pair feeding was made possible by removing the time restriction and they were allowed to eat an identical amount of food as the wild type animals. Notably, all the food was consumed within approximately 2.5 h in this group. Of interest, 2h and 3h but not 4h food restriction in female C57Bl/6 mice of the approximate same age as the mice used here induces a robust FAA and anorexic response on day 1-3 of the scheduled feeding

(Kas et al., 2003). Food intake was monitored daily and body weights were recorded before the onset of the dark phase. Due to limitations within the ethical permissions regarding maximum body weight loss, the experiment was ended following 3 days of scheduled feeding.

Localization of injections

Series of 16 μm coronal sections of the brain were sliced using a cryostat (Leica, Rijswijk, The Netherlands), thaw-mounted onto RNAse free Superfrost slides (Menzel, Germany) and stored at -80ºC until processing. For the localization of ICV injections, brain sections (16 μm) were stained with Cresyl violet. Cannula placement was defined appropriate when positioned in the lateral ventricle, based on Paxinos brain atlas (Paxinos and Watson, 1998). Rats with incorrect cannula placements were removed from further analysis.

Statistical analysis

All data are expressed as mean ± standard error. In order to measure plasma ghrelin levels during the course of the ABA model, groups of rats were decapitated at consecutive days. Therefore, average daily running wheel activity and relative body weight values were based on varying number of individuals in the running group (varying from 29 till 4 rats) and pair-fed group (varying from 24 till 4 rats) over time. On day 4 and 5 of this experiment, association between physiological parameters and the development of hyperactivity in the light phase was investigated using Pearson's bivariate correlation analysis. We analyzed possible associations between plasma ghrelin

levels and food anticipatory activity on day 4 and 5 while on these days hyperactivity developed in the light phase. All data were analyzed using SPSS 11.5 for Windows, using ANOVA with Bonferoni *post hoc* testing or Student's *t*-tests when appropriate. Statistical significance was set at $p \leq 0.05$.

3. RESULTS

Physiological parameters during the development of ABA in rats

Baseline body weight during *ad libitum* feeding was not significantly different between rats in running wheel cages and sedentary rats (213.7 ± 1.4 gram versus 218.9 ± 2.8 gram respectively). Running rats had a higher baseline food intake as compared to sedentary rats (19.7 ± 0.3 gram versus 18.1 ± 0.4 gram respectively, p=0.001), that likely reflects a compensation for the increased physical activity. During food restriction, body weight decreased in both ABA rats and pair-fed sedentary rats. ABA rats had a lower relative body weight compared to pair-fed sedentary rats (day 1 to day 4, $p<0.001$; day 5, p=0.008; Figure 2). Just prior to day 5, the body weight of the ABA rats decreased remarkably (Figure 2).

Endocrine parameters during the development of ABA in rats

During exposure to the ABA model, plasma ghrelin levels increased over time ($F_{(5,28)}=4.50$, p=0.01) whereas no significant changes were observed for plasma ghrelin levels in pair-fed sedentary rats ($F_{(5,23)}=2.12$, Table 1).

Association between plasma ghrelin levels and hyperactivity in rats

Our food anticipation studies are built upon the well-documented observation that animals become more active during hours preceding food access. Based on a review by Mistlberger (Mistlberger, 1994), we defined food anticipatory activity as the running wheel activity during the four hours prior to food access on the days of scheduled food

access. Since, under restriction conditions food was given during the first hour of the dark phase, the running wheel activity in the last four hours of the light phase was taken into consideration for the evaluation of food anticipatory activity.

Increased activity, especially prior to food intake, is a hallmark feature of ABA and comparable to hyperactivity observed in patients suffering from anorexia nervosa (Hillebrand et al., 2008). We therefore sought to determine whether high plasma ghrelin levels were associated with food anticipatory activity. Data points from ABA day 4 and day 5 were combined, corresponding to a period when food anticipatory activity is usually fully expressed. Correlation analysis on day 4 and 5 revealed that plasma ghrelin levels were positively correlated with food anticipatory activity in ABA rats ($r=0.760$, $p=0.018$, Figure 3.A.). There was no correlation between plasma ghrelin levels and total running wheel activity on the same days (Figure 3.B.).

Effect of acute central injection of a GHS-R1A antagonist on food-anticipatory activity in rats

To investigate whether central ghrelin signaling contributes to the development of hyperactivity caused by caloric restriction, we injected a ghrelin receptor (GHS-R1A) antagonist centrally into the brain ventricles during *ad libitum* fed and food restricted conditions. As already indicated, the running wheel activity in the last four hours of the light phase was taken into consideration for the evaluation of food anticipatory activity. To circumvent the large variation in running wheel activity between rats within the same experimental group, food anticipatory activity is presented as percentage of total running

wheel activity during the same day of acute ICV injection with the GHS-R1A antagonist or saline vehicle. The %food anticipatory activity after ICV injection at the end of day 4 was compared to the %food anticipatory activity on the day before (the end of ABA day 3). As depicted in figure 4, acute injection with the highest concentration of the GHS-R1A antagonist (JMV2959 16 μg) in the lateral ventricle tended to decrease food anticipatory activity (p=0.06) whereas the lower concentrations had no effect.

Effect of acute central injection of a GHS-R1A antagonist on food intake in rats

In *ad libitum* fed rats, acute ICV injection of the GHS-R1A antagonist (16 μg) reduced daily food intake (Figure 5.A.). A tendency towards a decrease in food intake was observed using a slightly lower dose (8 μg) of the antagonist. Vehicle injection or injection with the lowest dose of the antagonist (4 μg) did not affect daily food intake. In ABA rats, acute ICV injection of the GHS-R1A antagonist had no effect on daily (restricted) food intake (Figure 5.B.). Thus, acute ICV injection of a GHS-R1A antagonist reduces food intake in *ad libitum* fed running rats, but not in ABA rats.

Lack of food-anticipatory activity in ghrelin receptor KO mice

To confirm the involvement of ghrelin signaling in food anticipatory activity found in rats, we exposed wild type and GHS-R1A KO mice to the ABA model. Since scheduled-fed GHS-R1A KO mice exhibited a reduced daily food intake as compared to the control wild type mice during the scheduled feeding (total 3 day food intake; wild type: 5.5 ± 0.4 gram, scheduled-fed GHS-R1A KO: 3.6 ± 0.5 gram, $p<0.001$, Student's *t*-test), we included an additional experimental group: GHS-R1A KO mice that were pair-fed to the

wild type mice. Food intake was not significantly different between the groups during the 5 day habituation period.

Over the whole period of scheduled food restriction, pair-fed GHS-R1A KO mice lost less body weight as compared to wild type and the scheduled-fed GHS-R1A KO mice ($p<0.01$, ANOVA followed by Bonferoni *post hoc* test, Figure 6.B.). (double checked and this is only true for the first run and not the repeat which yield a p value 0.15)

Because of severe body weight loss and the significant differences observed in body weight loss on ABA day 3, we analyzed running wheel activity levels on day 2 of the ABA model. Baseline running wheel activity was not significantly different between the groups during the 5 days habituation. During the course of the ABA model, running wheel activity in the dark phase remained similar between wild type, schedule-fed GHS-R1A KO, and pair-fed GHS-R1A KO groups. Wild type mice clearly developed food anticipatory activity (Figure 6.A.). Scheduled-fed GHS-R1A KO also developed a food anticipatory response however this was attenuated compared to wild type mice ($p<0.05$, Fig 6.A.). Day time running wheel activity of pair-fed GHS-R1A KO mice on day 2 was significantly decreased relative to both wild type and schedule-fed GHS-R1A mice (both $p<0.05$) and did not differ from the habituation when the animals had *ad libitum* access to food. Thus, lack of ghrelin signaling in GHS-R1A KO mice results in reduced anticipation to food.

Effects of chronic pharmacological suppression of central ghrelin signaling on food-anticipatory activity

To extend our acute studies showing that a GHS-R1A antagonist suppresses food anticipatory activity in the ABA model, an additional study was undertaken in which food anticipatory activity was measured in ABA mice following chronic peripheral administration of the same antagonist at two different doses, namely 6 and 12 mg/kg/day of JMV2959. Baseline running wheel activity (during habituation) did not differ between the JMV2959- and saline-treated groups. Similar to the analysis of food anticipatory activity in the GHS-R1A KO study, we analyzed food anticipatory activity on ABA day 2 (Figure 7). The highest dose of the antagonist (12 mg/kg/day), reduced food anticipatory activity without affecting night time running ($p<0.05$). Furthermore, body weight and food intake were unaffected by the GHS-R1A antagonist compared to vehicle treatment regardless of the dose (6mg/kg/day dose: BW, saline: 15.6 ± 0.3 gram JMV2959: 15.9 ± 0.4, food intake, saline: 0.65 ± 0.2 gram JMV2959: 0.49 ± 0.7 gram, 12mg/kg/day dose: BW, saline: 16.7 ± 0.3 gram JMV2959: 17.2 ± 0.2, food intake, saline: 2.5 ± 0.2 gram JMV2959: 2.3 ± 0.5 gram) . Thus, continuous peripheral administration of the GHS-R1A antagonist suppressed food anticipation without affecting the regulation of body weight or food intake.

4. DISCUSSION

In the present study we demonstrate that the central ghrelin signaling system, involving GHS-R1A, is required food anticipation, measured in the ABA model. Thus, food anticipatory activity was attenuated both in chronic models of suppressed ghrelin signaling (GHS-R1A KO mice and mice chronically treated with a GHS-R1A antagonist) and in an acute model (rats given an acute central injection of the GHS-R1A antagonist). Importantly, the effect on food anticipatory activity wase independent of food intake in both the acute and chronic studies using the GHS-R1A antagonist. Collectively these data suggest that the gut-brain signal provided by ghrelin is important for food anticipation and that GHS-R1A antagonists provide a potential therapy for suppressing food anticipatory hyperlocomotor activity independently of food intake.

Our data, showing suppressed food anticipatory activity in GHS-R1A KO mice, are in good agreement with recent papers on this topic (Blum et al., 2009;LeSauter et al., 2009). Rhythmic ghrelin and the circadian clock gene PER expression in stomach oxyntic cells are synchronized to timing of food delivery and GHS-R1A KO mice show less food anticipatory activity when food is given for 6h (LeSauter et al., 2009) or for 4h (Blum et al., 2009) in the light phase. In the present study, we compared GHS-R1A KO and wild type mice put on a 2h feeding schedule in the beginning of the dark phase which rapidly induces both a FAA and an anorexic response when combined with free access to running wheels. Given that young female GHS-R1A KO mice are not able to adapt their food intake on a 4h feeding schedule (Blum et al., 2009) we also included a third experimental

group, GHS-R1A KO mice that were pair fed to the wild type group (by increasing the amount of time exposed to the food). As food anticipatory activity was decreased in both the GHS-R1A KO group on a 2 hr feeding schedule and the pair-fed GHS-R1A KO group compared to wild type mice, it seems likely that the suppressed food anticipatory activity (i.e. hyperlocomotor activity) is not secondary to the caloric deficit and body weight loss, but rather, relates directly to suppressed GHS-R1A signaling. Furthermore, night time running activity was also similar between all three groups indicating that the changes in FAA found was not due to fatigue induced by differences in caloric intake. However, the decrease in FAA in the pair fed GHS-R1A KO group may be a consequence of the longer exposure to food *per se* rather than the lack of ghrelin signaling. Conversely, robust FAA has been reported on day 1-3 following the start of food restriction in mice of the same strain and age using either 2h or 3h scheduled feeding protocols (Kas et al., 2003).

Previously we reported that central administration of the ghrelin antagonist JMV2959 suppresses ghrelin-induced food intake (Salome et al., 2009a) and ghrelin-induced adiposity (Salome et al., 2009b). However, in the present study we were unable to detect any effects of the antagonist on food intake *per se*, in the dose range used to suppress food anticipatory response. Thus, we found that the highest dose of the GHS-R1A antagonist tested (16 µg JMV2959, ICV) reduced food intake in *ad libitum* fed running rats but had no effect on food intake in the ABA group. Similar effects on food anticipatory activity were also observed in rats upon acute administration of the antagonist as well as in a chronic study involving peripheral treatment of the same

antagonist (12 mg/kg/day) to mice exposed to the ABA model. Given that plasma ghrelin levels are associated with food anticipatory activity but not with total running wheel activity and also that the GHS-R1A antagonist suppresses food anticipatory activity, our data provide strong evidence that the central ghrelin signaling system (via GHS-R1A) is involved in food anticipation. This hypothesis is further supported by the aforementioned studies in which we show a lack of food anticipatory activity in the GHS-R1A KO mice exposed to the ABA paradigm. We may infer that GHS-R1A is not only involved in the development of food anticipatory activity (from studies in GHS-R1A KO mice) but that it is possible to manipulate food anticipatory activity using acute therapeutic intervention involving GHS-R1A antagonists.

In the present study we also demonstrate an association between plasma ghrelin levels and food anticipatory activity in the ABA model. Thus, in ABA rats there was a strong positive correlation between the expression of food anticipatory activity (i.e. running wheel activity during the four hours prior to scheduled food access) and plasma ghrelin levels on day 4 and day 5 of exposure to the ABA paradigm. Interestingly, plasma ghrelin levels were not correlated with total (24 hour) running wheel activity but only to the food anticipatory phase, supporting the idea that ghrelin's locomotor stimulatory activity may be especially linked to the food anticipatory period.

Studies employing Fos activity in GHS-R1A KOs have highlighted key hypothalamic areas that are likely to be of importance for ghrelin's effects on food anticipatory activity (Blum et al., 2009). Given that ghrelin also targets the midbrain dopamine system to

induce locomotor activity (Jerlhag et al., 2006;Jerlhag et al., 2007), further studies are required to discover whether this system is also important for ghrelin's role in food anticipatory (hyperlocomotor) activity. Moreover, it will be interesting to discover whether increased anticipatory activity contributes to ghrelin's effects to promote reward-seeking behaviour, not only for food but also for chemical drugs such as alcohol (Jerlhag et al., 2009).

Anorectic patients often display abnormally high physical activity levels (Kron et al., 1978), which hinders the process of recovery (Holtkamp et al., 2004;Kaye et al., 1988). Thus, reducing hyperactivity in severely ill patients suffering from anorexia nervosa could be beneficial for therapeutic outcome. The present data demonstrating that suppression of the central ghrelin signaling system (incorporating both pharmacological and genetic models) reduces hyperactivity without influencing food intake in the ABA paradigm suggests that the ghrelin receptor, GHS-R1A, could provide a therapeutically relevant target for treatment of anorexia nervosa. Interestingly, ghrelin infusion did not increase appetite in patients suffering from anorexia nervosa as compared to weight matched healthy controls (Miljic et al., 2006). In conclusion, our data support the hypothesis that suppression of the central ghrelin signaling system could suppress hyperactive behaviour in patients suffering from anorexia nervosa, based on rodent studies incorporating the ABA model.

DISCLOSURE/CONFLICT OF INTEREST

None of the authors has conflict of interests associated with the work reported in this paper.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Experimental set-up for the ABA model

Schema of experimental set-up. Filled bars indicate the dark phase, open bars the light phase. At day 0, food was removed for the restricted experimental groups and placed on scheduled feeding of 1-2 hour at the beginning of the dark phase. Food intake and body weight were measured daily.

Figure 2. Body weight changes in ABA rats and pair-fed sedentary rats

Daily body weight (as percentage of initial body weight, start of food restriction) in food-restricted running rats fed for 1 hour (black circle) and pair-fed sedentary rats (open circle). Significant differences between pair-fed sedentary and 1-hour fed running (ABA) rats are indicated by asterisk; ** $p<0.01$, *** $p<0.001$. Statistical significance was set at $p<0.05$.

Figure 3. Correlation between ghrelin levels and food anticipation

Correlation analysis on day 4 and 5 between plasma ghrelin levels and (A) total food-anticipatory activity (food anticipatory activity (FAA), measured by wheel revolutions) or (B) total running wheel activity levels in running rats exposed to 1-hour feeding schedule (ABA, n=9). Statistical significance was set at $p<0.05$.

Figure 4. Acute effect of GHS-R1A antagonist on food anticipation

Effect of acute ICV injection of saline (n=6) or the GHS-R1A antagonist, JMV2959, on food-anticipatory activity in rats exposed to the ABA model. On day 4, JMV2959 was administered at 3 different doses: 4 μg (n=10), 8 μg (n=6) and 16 μg (n=6). The percentage food-anticipatory activity was defined as the running wheel activity 4 h prior to food access as percentage of total running wheel activity on days of scheduled feeding. The white bars represent the mean percentage food-anticipatory activity on the day before ICV injection (ABA day 3). The black bars represent the mean percentage food-anticipatory activity just after ICV injection (ABA day 4). A tendency towards a significant reduction in percentage food-anticipatory activity was observed in rats with an acute ICV injection with JMV2959 16 μg ($p=0.06$).

Figure 5. Acute effect of GHS-R1A antagonist on food intake

Effect of an acute ICV injection of saline (n=6) or a GHS-R1A antagonist administered using three different doses just prior to day 5 on 24-hour food intake: 4 μg (*ad libitum* fed n=6; food-restricted n=10), 8 μg (*ad libitum* fed n=10; food-restricted n=6), or 16 μg (*ad libitum* fed n=8; food-restricted n=6). (A) *ad libitum* fed running rats and (B) rats exposed to the ABA model. Food intake is presented in absolute values. Significant differences are indicated by asterisks, *$p<0.05$, Student's *t*-test.

Figure 6. Activity levels and body weight changes in ghrelin ko mice exposed to the ABA model.

Running wheel activity (A) and relative body weights (B) in wild type mice (white circle, n=6), schedule-fed ghrelin receptor (GHS-R1A) knockout (KO) mice (black circle, n=6),

and pair-fed GHS-R1A KO mice (gray circle, n=7) exposed to the ABA model. Black bars indicate dark phase, and white bars indicate light phase. Hourly running wheel activity is presented as mean ± SEM, and body weights are plotted as percentage of initial body weight. Differences in running wheel activity during the dark phase or light phase was analyzed using ANOVA repeated measures followed by Bonferoni *post hoc* testing. Significant differences between wild type and schedule-fed GHS-R1A KO mice are indicated by "a", whereas "b" indicates significant differences between wild type and pair-fed GHS-R1A KO mice. Statistical significance was set at $p<0.05$.

Figure 7. The effect of continuous peripheral administration of GHS-R1A antagonist on activity levels in ABA mice.

Hourly running wheel activity on day 2 of the ABA model in mice (A) treated with JMV2959 6 mg/kg/day (black circle, n=6) and (B) treated with JMV2959 12 mg/kg/day (black circle, n=6) as compared to mice treated with saline solution (white circle, n=6 and n=5 respectively). Filled black bars indicate the dark phase, open bars the light phase. Differences in running wheel activity during the dark phase or light phase was analyzed using ANOVA repeated measures followed by Bonferoni *post hoc* testing. Significant differences between wild type and GHS-R1A antagonist-treated mice are indicated by "a". Statistical significance was set at $p<0.05$.

Table. 1. Plasma ghrelin levels during scheduled feeding

	plasma ghrelin levels (ng/ml)	
day	1-hour fed running rats	pair-fed sedentary rats
0	0.80 ± 0.05	1.13 ± 0.15 [b]
1	2.10 ± 0.18	2.61 ± 0.40
2	2.28 ± 0.44	2.23 ± 0.57
3	3.64 ± 1.43	2.19 ± 0.54
4	2.74 ± 0.36	1.45 ± 0.19 [b]
5	5.10 ± 0.35 [a]	2.55 ± 0.28

Total plasma ghrelin levels in 1-hour fed running rats (ABA) and pair-fed sedentary rats. Significant differences within the experimental groups compared to day 0 are indicated by “a”, significant different from ABA rats at the same day are indicated by “b”. ANOVA, Bonferroni, with significance set at $p<0.05$.

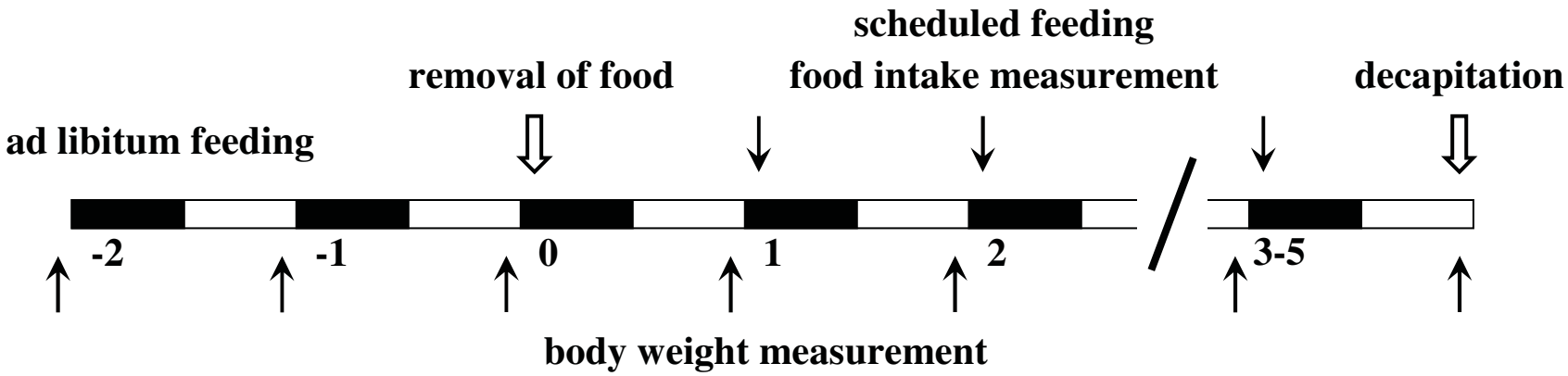

scheduled feeding
removal of food
food intake measurement
decapitation
ad libitum feeding
-2
-1
0
1
2
3-5
body weight measurement

Figure 2

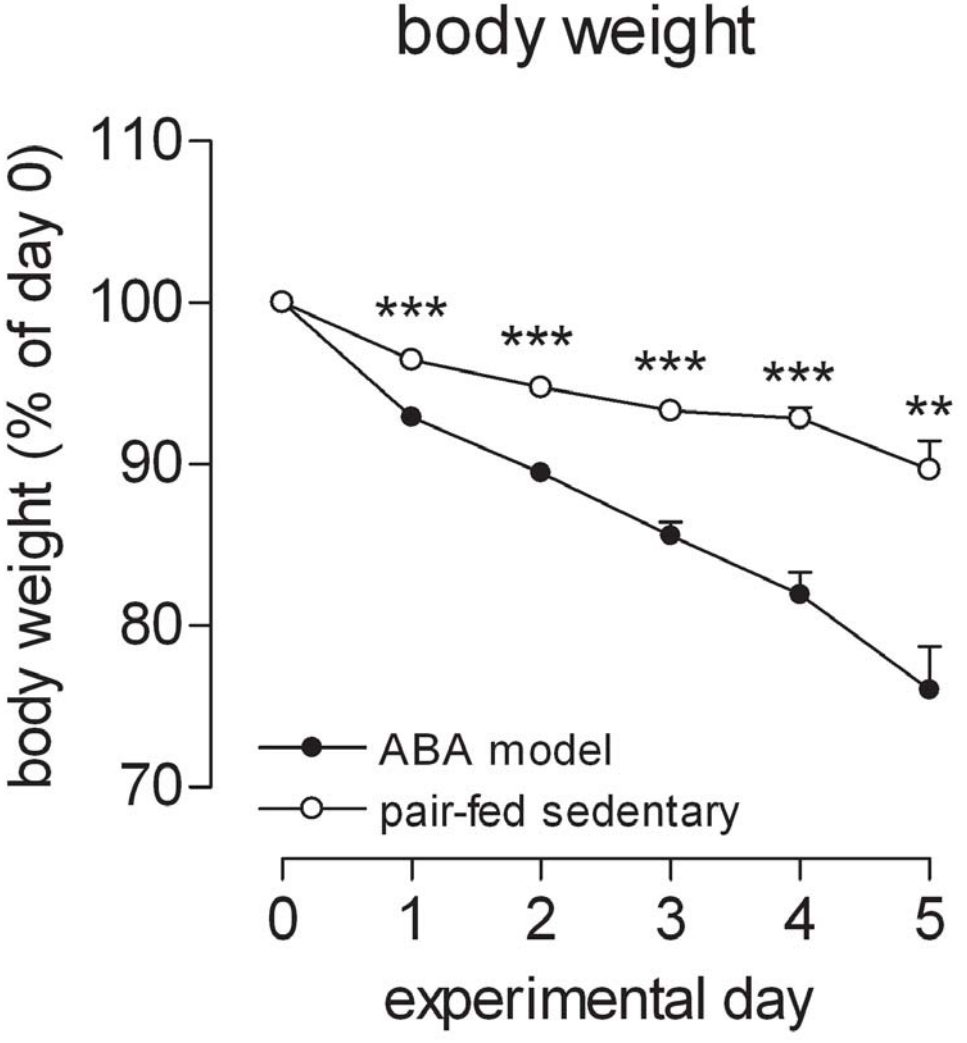

body weight
body weight (% of day 0)
110
100
90
80
70
***
***
***
***
**
ABA model
pair-fed sedentary
0
1
2
3
4
5
experimental day

Figure 3

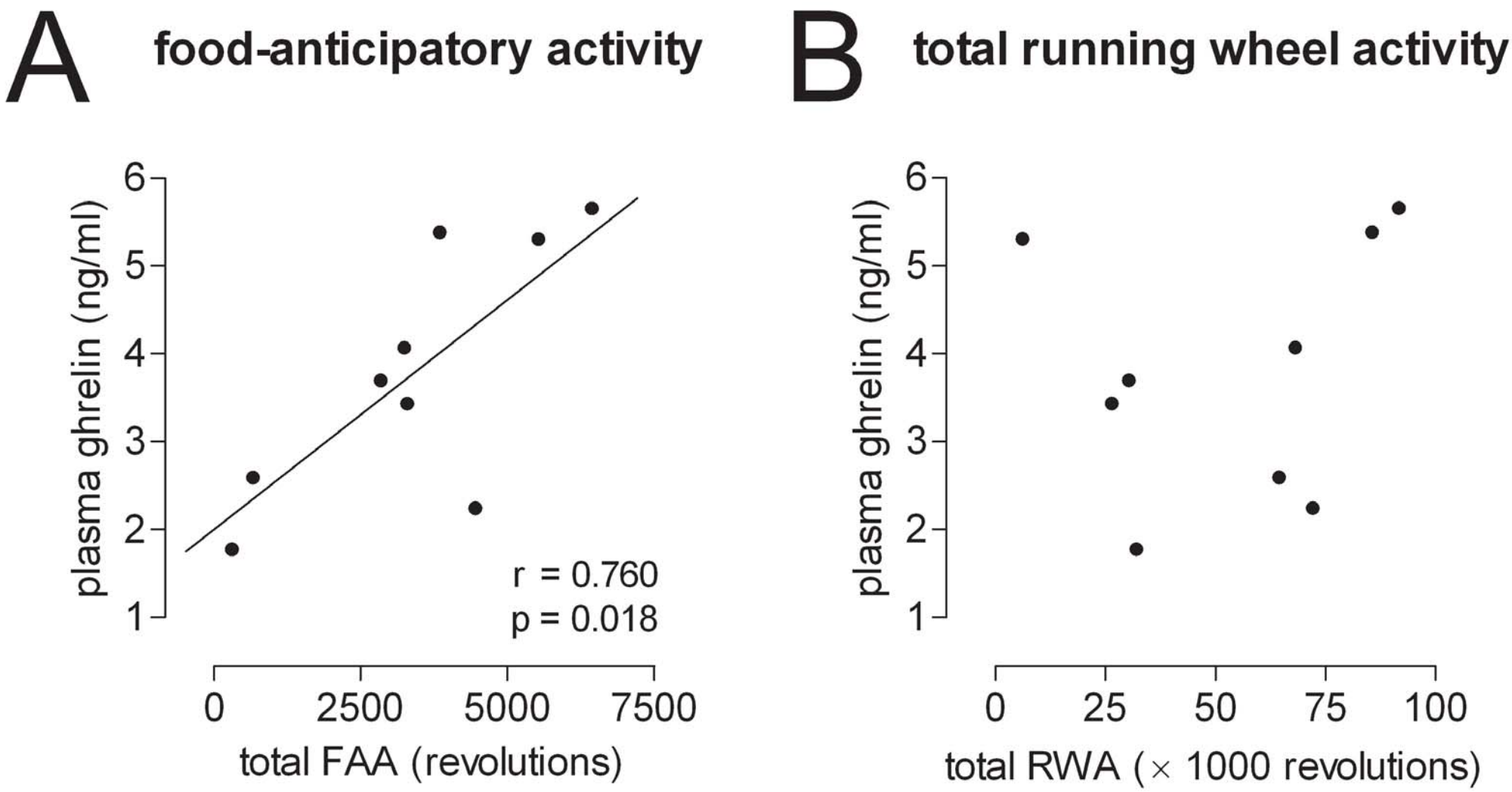

A
food-anticipatory activity
plasma ghrelin (ng/ml)
1
2
3
4
5
6
r = 0.760
p = 0.018
0
2500
5000
7500
total FAA (revolutions)
B
total running wheel activity
plasma ghrelin (ng/ml)
1
2
3
4
5
6
0
25
50
75
100
total RWA (× 1000 revolutions)

Figure 4

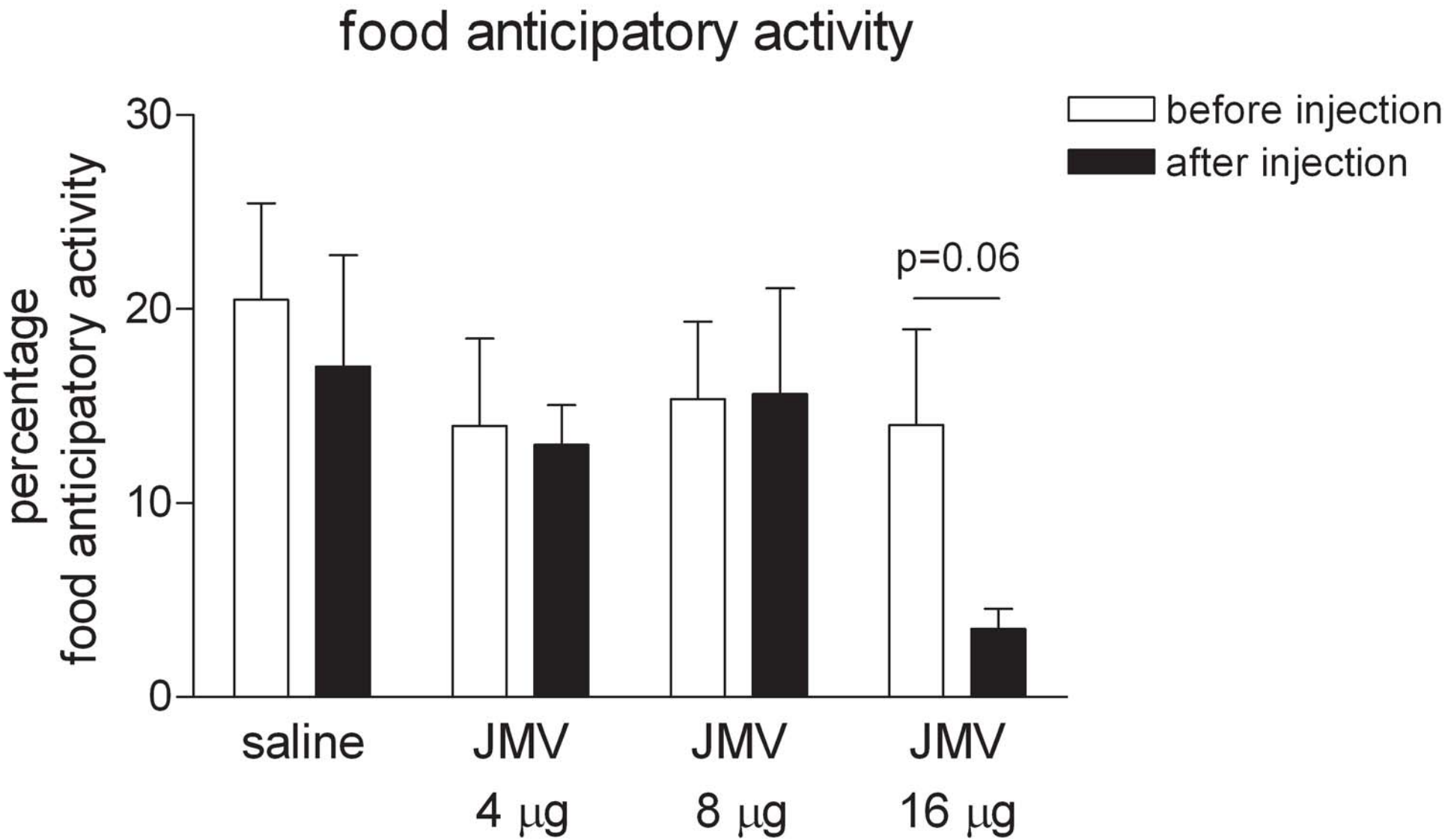

food anticipatory activity
before injection
after injection
percentage food anticipatory activity
30
20
10
0
p=0.06
saline
JMV 4 μg
JMV 8 μg
JMV 16 μg

Figure 5

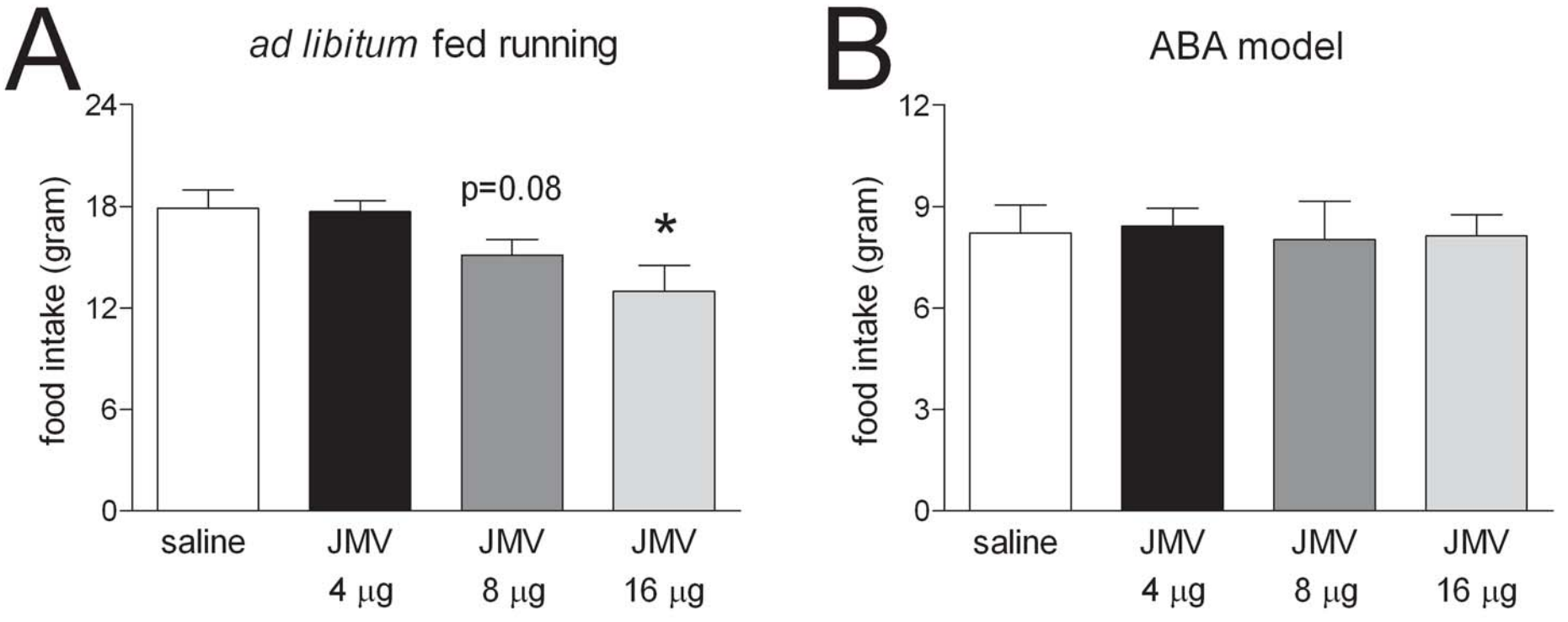

A
ad libitum fed running
food intake (gram)
24
18
12
6
0
p=0.08
*
saline
JMV 4 μg
JMV 8 μg
JMV 16 μg
B
ABA model
food intake (gram)
12
9
6
3
0
saline
JMV 4 μg
JMV 8 μg
JMV 16 μg

Figure 6

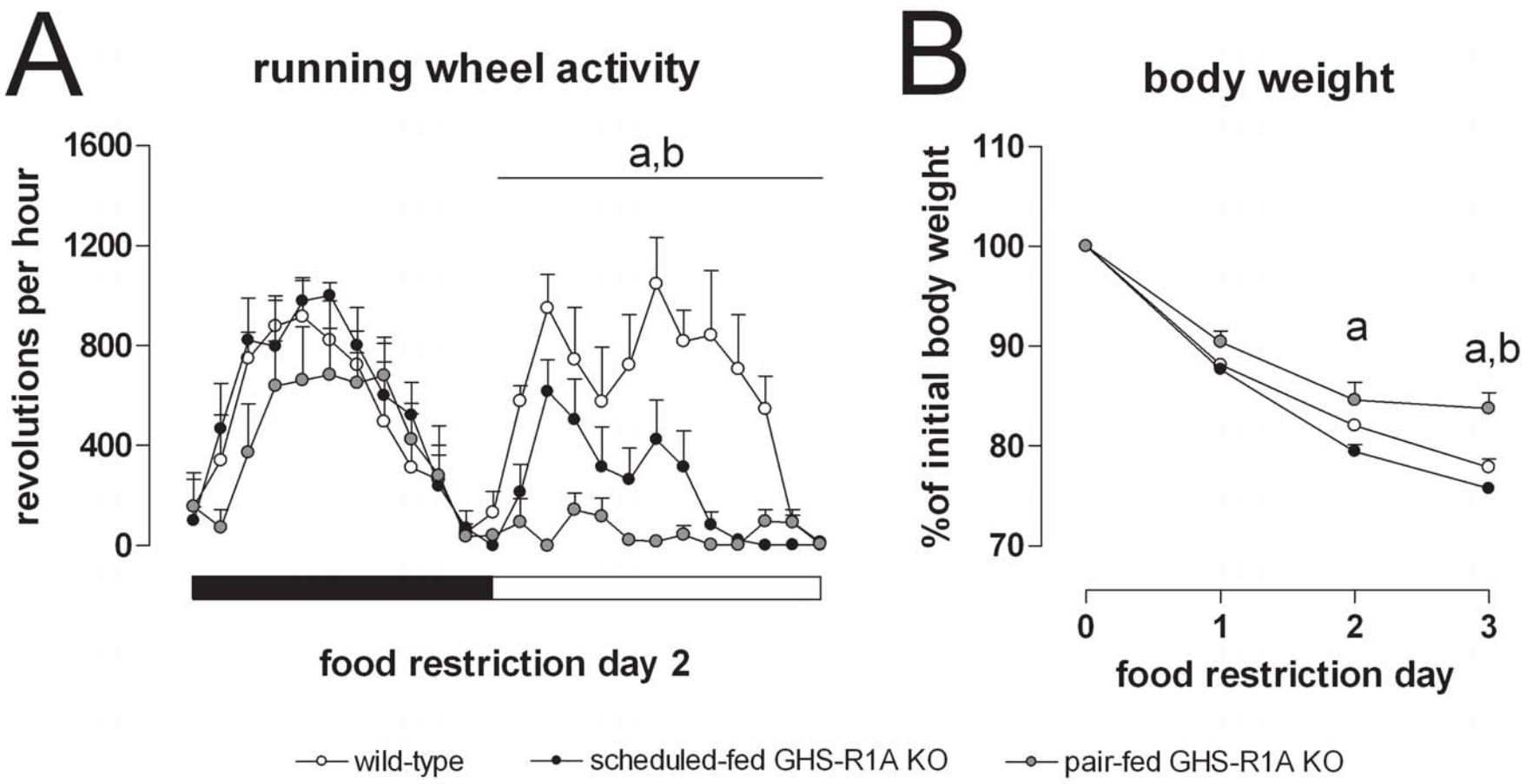
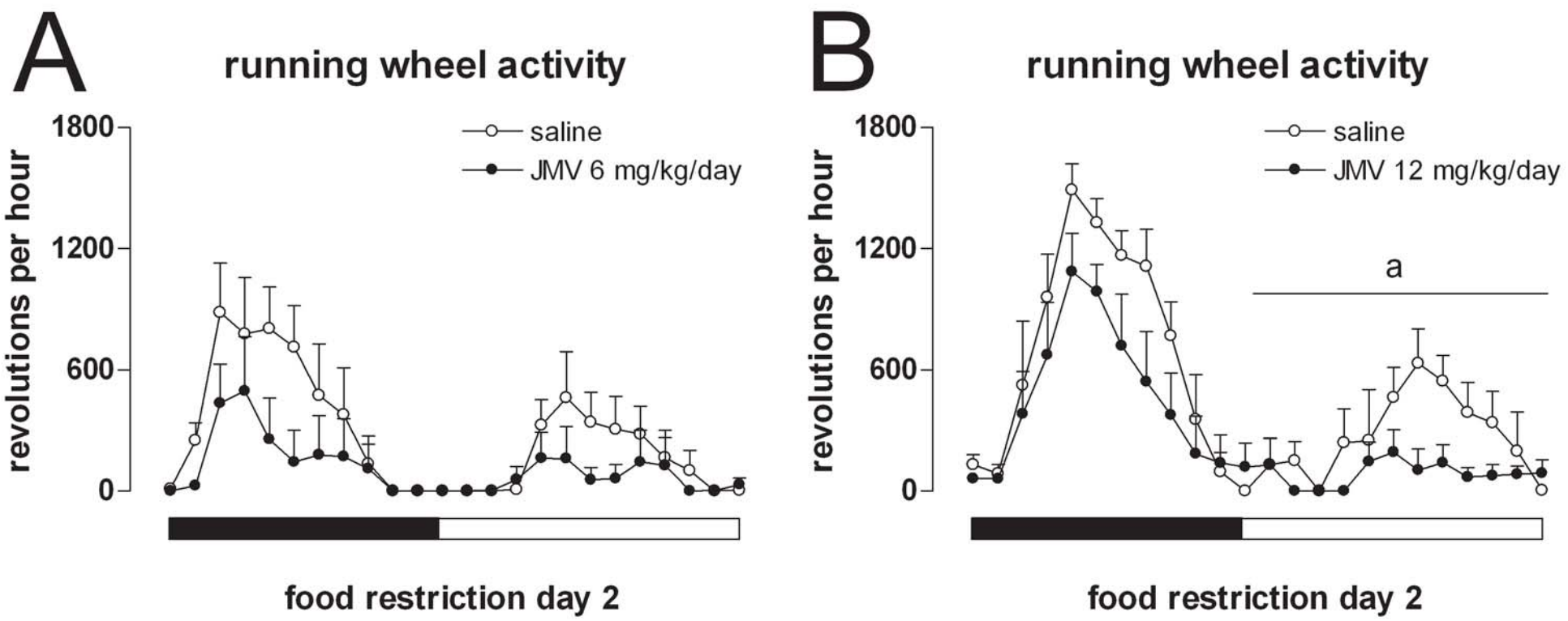

A
running wheel activity
1600
1200
800
400
0
revolutions per hour
a,b
food restriction day 2
B
body weight
110
100
90
80
70
%of initial body weight
a
a,b
0
1
2
3
food restriction day
wild-type
scheduled-fed GHS-R1A KO
pair-fed GHS-R1A KO

Figure 7


A
running wheel activity
1800
1200
600
0
revolutions per hour
saline
JMV 6 mg/kg/day
food restriction day 2
B
running wheel activity
1800
1200
600
0
revolutions per hour
saline
JMV 12 mg/kg/day
a
food restriction day 2