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NEUROSYSTEMS

# Altered metabolic and neurochemical responses to chronic unpredictable stressors in ghrelin receptor-deficient mice

Z. R. Patterson, R. Ducharme, H. Anisman and A. Abizaid
Institute of Neuroscience, Carleton University, 1125 Colonel By Drive, Ottawa, K1S 5B6 ON, Canada

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## Abstract

Ghrelin, a hormone produced by the stomach, is generally associated with feeding responses and the regulation of food intake. Recent evidence, however, suggests that ghrelin is also a stress hormone, given that it is released following acute and chronic stressors. The present study examined the role of ghrelin in producing normal metabolic and neurochemical responses to chronic stress. This was achieved by examining these responses in mice with targeted deletions of the ghrelin receptor gene (GHSR KO mice), and comparing them with the same responses in their wild-type (WT) littermates. As expected, WT stressed mice decreased their caloric intake, body weight gain and caloric efficiency while maintaining adiposity. GHSR KO mice, however, did not show these alterations despite having normal glucocorticoid responses to stress. In parallel with these changes, chronic unpredictable stress caused changes in norepinephrine, dopamine and serotonin in a number of brain regions. Of these, norepinephrine neurotransmission in the arcuate nucleus and prefrontal cortex was differentially altered in GHSR KO mice. Within the nucleus acumbens, dopamine utilization was increased in WT mice but not in GHSR KO mice. Finally, there were strain differences in serotonin neurotransmission that may explain interstrain body weight and adiposity differences. These results suggest that the metabolic changes necessary to deal with the energetic challenge presented by repeated exposure to stressors do not occur in GHSR KO mice, and they are discussed within the context of the potential vulnerability to stress-induced pathology.

## Introduction

Stress is viewed as a state in which behavioral and physiological responses are generated in the face of a perceived threat. The physiological signatures of this state are sympathetic nervous system and hypothalamic–pituitary adrenal (HPA) axis activation as well as variations of several neurotransmitters within the brain (McEwen, 2007; Ulrich-Lai & Herman, 2009). Acutely, these responses are considered to be adaptive, but continuous stressor exposure may lead to physiological alterations that ultimately result in detrimental health effects, including major depressive illness, type II diabetes, obesity and cardiovascular disease (Frasure-Smith & Lesperance, 2005; Black, 2006; McEwen, 2007; Anisman *et al.*, 2008).

A physiological consequence of chronic stressors is a change in metabolism that entails an increase of metabolic rate, where proteins become inappropriately metabolized while fat stores are spared (Depke *et al.*, 2008). These stressor-induced metabolic changes are generally attributed to continuous stimulation of both the sympathetic system and the HPA axis (Dallman *et al.*, 2007; Kuo *et al.*, 2008). Persistent increases in glucocorticoids have, in particular, been linked to metabolic and behavioral abnormalities including obesity, compromised immunity and depression (Dallman *et al.*, 1993, 2006, 2007; Black, 2006; Bartolomucci *et al.*, 2009).

*Correspondence*: Dr A. Abizaid, as above.
E-mail: alfonso_abizaid@carleton.ca

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Ghrelin, a hormone produced by the stomach, is associated with the regulation of appetite and metabolism (Kojima *et al.*, 1999; Tschop *et al.*, 2000; Nakazato *et al.*, 2001; Kirchner *et al.*, 2009). Continuous peripheral or central ghrelin infusions increase food intake and body weight, and favor the utilization of carbohydrates while preventing the utilization of body fat as a fuel substrate, ultimately leading to increased adiposity (Tschop *et al.*, 2000). This would be helpful during stress, where defensive responses require the availability of energy from substrates that can be oxidized rapidly, while maintaining fat depots (Kyrou & Tsigos, 2007). Indeed, in parallel with corticosterone, circulating plasma ghrelin levels increase in response to acute and chronic stressors (Asakawa *et al.*, 2001; Kristenssson *et al.*, 2006; Ochi *et al.*, 2008; Zheng *et al.*, 2009). Ghrelin also affects the activity of ascending monoaminergic neurotransmitter systems that are activated in response to stressors (Brunetti *et al.*, 2002; Date *et al.*, 2006; Nonogaki *et al.*, 2006; Kawakami *et al.*, 2008). Indeed, noradrenergic cells in the brainstem nucleus of the solitary tract (NTS), dopaminergic cells in the midbrain ventral tegmentum and substantia nigra (VTA/SN), and serotonergic cells in the midbrain raphe are all sensitive to ghrelin (Guan *et al.*, 1997; Mitchell *et al.*, 2001; Carlini *et al.*, 2004; Abizaid *et al.*, 2006; Date *et al.*, 2006; Zigman *et al.*, 2006) and modulate the activity of hypothalamic and limbic structures associated with stressor-induced pathology (Hoebel *et al.*, 1989; Anisman & Zacharko, 1990; Bremner *et al.*, 1996).

Given the links between stressors and both ghrelin and central monoamine variations, we assessed the importance of ghrelin in mediating stressor-provoked metabolic and neurotransmitter changes.

To do this, we exposed mice with genetic deletions to the ghrelin receptor gene (GHSR KO) to chronic unpredictable stressors for 2 weeks (Tannenbaum *et al.*, 2002), and compared their caloric intake, body weight gain and adipose tissue size with those of their wild-type (WT) littermates. We also examined differences in the content of norepinephrine, dopamine and serotonin and their metabolites in several brain regions affected directly or indirectly by ghrelin and by stress.

## Materials and methods

### *Animals*

Male GHSR WT ($n$ = 20) and male GHSR KO ($n$ = 20) mice, 2–4 months old that had been bred at Carleton University's Institute of Neuroscience, served as experimental subjects. These mice were created on a C57BL/J6 and DBA mixed background strain and originated from breeding pairs obtained from Regeneron Pharmaceuticals Inc. (Tarrytown, NY, USA). Their metabolic phenotype has previously been characterized (Pfluger *et al.*, 2008). All animals were single-housed in standard plastic mouse cages (27 × 21 × 14 cm) in a temperature-controlled room (22°C), and kept on a 12-h light–dark cycle with lights on at 07:00 h for the duration of the study. Stressed animals were housed in a different room from non-stressed animals. All procedures were approved by the Carleton University Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care.

### *Procedure*

Animals were matched for age (average age 82.8 ± 2.3 days old) and body weight (30.1 ± 1.13 g) and were assigned to one of four groups: (i) WT no stress ($n$ = 10), (ii) WT stress ($n$ = 10), (iii) KO no stress ($n$ = 10) and (iv) KO stress ($n$ = 10). Standard and high-fat diet intake and body weight were measured for 10 days before the onset of the unpredictable stressor regimen to obtain a baseline. At the end of the baseline period, WT and GHSR KO mice in the stress groups were taken to a separate room and subjected to two daily stressors for 14 days. The stressors used were as follows. (i) Restraint: mice were placed in a semicircular chamber strainer made of clear Plexiglas (4.0 cm diameter × 12.0 cm long), for 15 min with their tails taped to prevent them from turning. (ii) Exposure to predator scent: mice were placed in a rat cage (32 × 22 × 20 cm) with soiled litter for 15 min. (iii) Novel aversive environment: mice were placed in a clear mouse cage (27 × 21 × 14 cm) with wet bedding for 15 min. (iv) Social stressor: mice were placed in the cage of a sexually experienced male mouse (a mouse of the highly aggressive CD-1 strain) for a maximum of 15 min, or until fighting occurred. This interaction was always stopped before experimental mice were physically injured. (v) Forced swim test: mice were placed in a pool of water (32 × 22 × 20 cm) at 20°C for 3 min. (vi) Noise stress: mice were kept in their home cages and exposed to loud music for 15 min. (vii) Open field stress: mice were placed in the center of an open field box (60 × 60 × 20 cm) for a period of 15 min. Animals were exposed to a different stressor (chosen randomly) and at two randomly selected times of the day. In this way, mice were not able to adapt to a single stressor nor were they able to predict when they were to be stressed. This combined stressor paradigm has been used previously and shown to be effective in producing marked hormonal and neurochemical responses (Shanks *et al.*, 1994; Mineur *et al.*, 2003, 2006, 2007). We emphasize that this regimen involves stressors that are more intense than those typically used in chronic mild stress paradigms (Willner *et al.*, 1987).

Following the 14th day of treatment, mice from both groups were killed by rapid decapitation 5 min after the last stressor (this being 15 min of restraint stress). Control non-stressed mice were killed on the same day as stressed animals, and occurred between 08:00 and 12:00 h. Trunk blood was collected in borosilicate tubes coated with EDTA and containing 10 μL of 1 N HCl per milliliter of blood and a cocktail of protease inhibitors and chilled on ice before being centrifuged. Brains were rapidly dissected and placed on a stainless steel block (immersed in ice) with slots (spaced approximately 500 μm apart) that served as guides for razor blades. Tissue micropunches of the hippocampus (1.5–3.0 mm behind bregma), central nucleus of the amygdala (1.0–1.5 mm behind bregma), paraventricular nucleus of the hypothalamus (PVN) (0.5–1.0 mm behind bregma), arcuate nucleus of the hypothalamus (ARC) (1.5–2.0 mm behind bregma), prefrontal cortex (1.0–1.5 mm in front of bregma), locus coeruleus (5.3–5.8 mm behind bregma) and the nucleus accumbens (1.0–1.5 mm in front of bregma) were collected from slices using hollow 16- and 20-gauge needles with a beveled tip as described by Palkovits (1973). Paxinos & Franklin (2001) was used as a guide. Tissue punches were placed in 0.3 M monochloroacetic acid containing 10% methanol and internal standards, and stored at −80°C until assayed. Following decapitation, mice bodies were collected, wrapped in aluminum foil and frozen at −80° until fat dissection was possible. The carcasses were later thawed and retroperitoneal, perigonadal and subcutaneous fat pads were dissected and weighed. The mass of adipose tissue in these fat pads was converted to a percentage of total body weight for statistical analyses.

### *Caloric intake and body weight*

Mice were given free access to standard lab diet (18% caloric content from fat; 3.0 kcal/g; Harlan, Misississaga, ON, Canada), a high-fat diet (58% caloric content from fat, 5.56 kcal/g; Research Diets, New Brunswick, NJ, USA) and tap water throughout the study. Measurements of weight and caloric consumption for both standard lab diet and high-fat diet were recorded over a 10-day baseline period and during a 14-day stressor period. Recordings were carried out at the same time every day. Caloric value for each food type was used to calculate the total daily caloric intake for each mouse across both the baseline and the treatment periods, as well as the percentage of total calories consumed coming from each food type. Caloric efficiency, defined as the amount of weight gained per calorie of energy consumed during a given time period, was calculated for each mouse during the baseline period as well as the treatment period.

### *Hormone level analyses*

Blood samples were centrifuged at 3000 ***g*** for 5 min at 4°C to separate plasma. The plasma was then collected and stored at −20°C. Plasma acylated ghrelin and corticosterone levels were measured in duplicate using commercially available radioimmunoassay (RIA) kits [corticosterone (ICN Biomedicals, CA, USA), sensitivity ranging from 5.0 to 40.0 μg/dL; acylated ghrelin (Millipore, MA, USA), sensitivity ranging from 7.9 to 1000 pg/mL]. The RIA assay for acylated ghrelin detects with 100% cross-reactivity the active form of ghrelin (acylated ghrelin) in plasma samples from humans, rodents and canine species. Furthermore, the antibody detects specifically the first three aminoacids of the ghrelin molecule, including the Octanoyl group that is acylated to the serine 3 aminoacid in the sequence. This particular

portion is shared by most vertebrates (Hosoda *et al.*, 2006). All samples were assayed in a single run for each hormone and had an inter-assay variability lower than 10%.

### *Brain monoamine analyses*

Brain samples from all regions described above were homogenized and monoamine neurotransmitter analyses were performed from each independent sample using high-performance liquid chromatography (HPLC), as previously described (Anisman & Zacharko, 1990). Levels of dopamine (DA), norepinephrine (NE) and serotonin (5-HT), and their metabolites, 3,4-dihydroxy-phenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylglycol (MHPG) and 5-hydroxyindolacetic acid (5-HIAA), respectively, were assessed in each of the brain regions. To this end, tissue punches were sonicated in a homogenizing solution comprising 14.17 g monochloroacetic acid, 0.0186 g EDTA, 5.0 ml methanol and 500 mL HPLC-grade water. Following centrifugation, supernatants were used for the HPLC analysis. Using an Agilent pump (Mississauga, Ontario, Canada), guard column, radial compression column (5 m, C18 reverse phase, 8 mm × 10 cm), and colometric electrochemical detector (ESA), 40 $\mu$l of the supernatant from each individual region sampled from each mouse was passed through the system at a flow rate of 1.5 mL/min (1400–1600 pounds per square inch). Each liter of mobile phase contained sodium dihydrogen phosphate (90 mM), 1-octase sulfonic acid (sodium sal) (1.7 mM), EDTA (50 mM), citric acid (50 mM), potassium chloride (5 mM) and 10% acetonitrile. The mobile phase was filtered (0.22 mm filter paper) and degassed. The area and height of the peaks were determined using an Agilent data integrator and the software associated with this instrument. The protein content of each sample was determined using bicinchoninic acid with a protein analysis kit (Pierce Scientific, Brockville, ON, Canada) and a Fluorostar colorimeter (BMG, Durham, NC, USA). The lower limit of detection for the monoamines and metabolites was approximately 1.0 pg.

### *Statistical analyses*

All data were analysed using 2 × 2 ANOVAS with genotype (KO and WT) and treatment (stress or no stress) as the between-group factors. Tukey's HSD *post hoc* tests were performed where significant effects were found. The limit for statistical significance was set at an $\alpha = 0.05$.

## Results

### *GHSR KO mice do not show the stressor-induced alterations in caloric intake and body weight seen in GHSR WT mice*

Figure 1 shows the mean change in caloric intake during the treatment period as compared with baseline. The chronic stressor caused a significant decrease in caloric intake in WT stressed animals, but a similar effect was not observed in GHSR KO stressed mice [significant Stressor treatment × Genotype interaction ($F_{1,34} = 9.16$, $P < 0.05$)]. Similarly, body weight gain and caloric efficiency were lowered in WT stressed but not in GHSR KO stressed mice [significant Stressor treatment × Genotype interactions (weight gain: $F_{1,34} = 5.86$, $P < 0.05$; caloric efficiency: $F_{1,34} = 8.48$, $P < 0.05$)]. Interestingly, there were no selective differences in the proportion of calories obtained from either the palatable high-fat diet or the standard chow ($P > 0.05$), suggesting that the drop in caloric intake seen in WT mice comes from a drop in the consumption of both diets.

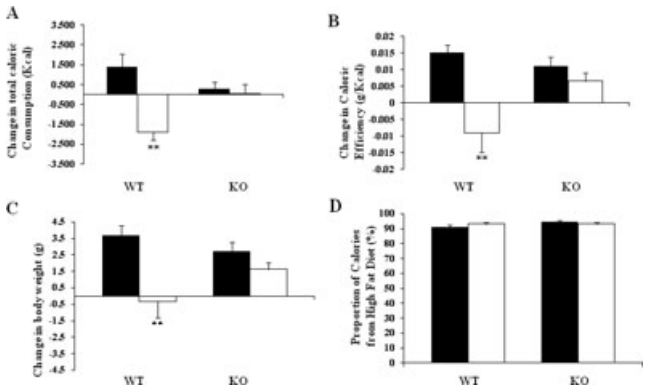


FIG. 1. Stress-induced changes from baseline in (A) caloric intake (± SEM), (B) caloric efficiency (means ± SEM), (C) weight change (means ± SEM) and (D) proportion of calories obtained from the consumption of a high-fat diet (means ± SEM) during the treatment period for non-stressed (black bars) and stressed (white bars) GHSR KO and wild-type mice. $^{**}P < 0.05$ compared with all other groups ($n = 10$ mice per group).

Figure 2 shows the proportion of body weight stored as fat in different fat pads. Despite a significant decrease in caloric intake, WT mice had an overall larger proportion of body weight stored in the fat pads than did GHSR KO mice (main effect of Genotype, $F_{1,34} = 94.03$, $P < 0.05$). Thus, WT mice maintained their total percentage of body fat and tended to have the highest percentage of abdominal fat [retroperitoneal + perigonadal, main effect of genotype ($F_{1,34} = 101.09$, $P < 0.05$)]. Similarly, WT mice tended to have more abdominal fat in relation to body weight than did GHSR KO mice and the stressor did not affect this proportion [main effect of genotype ($F_{1,34} = 56.97$, $P < 0.05$)].

Analysis of subcutaneous fat indicated that WT mice had higher proportion of subcutaneous fat than GHSR KO mice (Fig. 2C; $F_{1,34} = 56.97$, $P < 0.05$), but stress had no overall effect on the proportion of subcutaneous fat ($F_{1,34} = 0.296$, $P = 0.591$), and did not affect WT GHSR KO mice differentially (stress × genotype interaction, $F_{1,34} = 0.154$, $P = 0.699$).

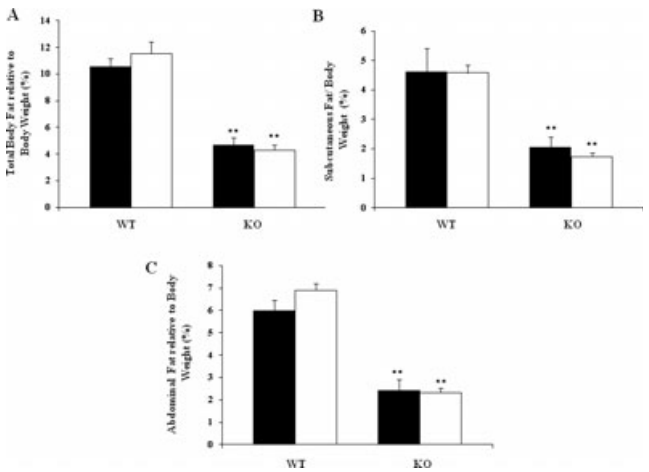


FIG. 2. Stress-induced alterations at the end of the stress paradigm in (A) total percentage body fat/total body weight (means ± SEM), (B) percentage abdominal fat/total body weight (± SEM) and (C) percentage subcutaneous fat/total body weight (means ± SEM) in non-stressed (black bars) and stressed (white bars) GHSR KO and wild-type mice. $^{**}P < 0.05$ compared with WT mice ($n = 10$ mice per group).

Figure 3 shows the plasma ghrelin and corticosterone levels measured from trunk blood samples taken at the time of death. The analyses revealed that stressed mice had significantly higher plasma active ghrelin ($F_{1,34} = 5.04$, $P < 0.05$) and cortisol levels ($F_{1,34} = 194.56$, $P < 0.001$). Moreover, WT animals showed overall higher levels of corticosterone then did KO mice ($F_{1,34} = 5.56$, $P < 0.05$).

### *Aminergic neurotransmitter concentrations are differentially altered by stress in GHSR KO mice*

Table 1 shows a summary of the significant main effects detected by the ANOVAS examining brain differences in the content of aminergic neurotransmitters and their metabolites in stressed and non-stressed GHSR KO and WT mice. All interaction effects are described in detail below. Non-significant interaction effects ($P > 0.05$) are not shown.

#### *NE and MHPG*

Statistical analyses showed that total NE concentrations were not significantly affected by the chronic stressor paradigm in any of the regions examined ($P > 0.05$). The chronic stressor, however, did increase the accumulation of MHPG in the central nucleus of the amygdala, hippocampus, prefrontal cortex and the hypothalamic PVN (see Table 1). Within the prefrontal cortex the stress-induced increase in MHPG was greater in GHSR KO mice than in WT mice as shown by a significant interaction effect ($F_{1,34} = 4.56$ and 5.22, $P < 0.05$; Fig. 4). Interestingly, MHPG concentrations in the ARC of stressed GHSR WT mice were lower than those in non-stressed WT mice, whereas in the ARC of stressed GHSR KO mice MHPG concentrations were increased (significant interaction effect, $F_{1,34} = 5.22$, $P < 0.05$; Fig. 4).

#### *DA, homovanillic acid (HVA) and DOPAC*

Overall, the chronic stressor paradigm was effective in altered DA utilization in some of the regions examined, as reflected by increases in the levels of DA metabolites but not DA. Specifically, the stressor increased the DA content in the nucleus accumbens (main effect of stress: $F_{1,34} = 3.98$, $P = 0.05$), and also increased DOPAC accumulation ($F_{1,34} = 7.97$, $P < 0.05$). Interestingly, the stressor increased DOPAC concentrations in WT mice but not in GHSR KO mice (significant genotype x stress interaction, $F_{1,34} = 5.19$, $P < 0.05$; Fig. 5). Finally, the stressor also increased DOPAC levels in the prefrontal cortex ($F_{1,34} = 5.60$, $P < 0.05$), but it did so to all animals irrespective of their genotype.

#### *5-HT and 5-HIAA*

5-HT concentrations in stressed and non-stressed GHSR KO and WT mice were not different in any of the areas examined with the exception of the ARC, where 5-HT levels were significantly lower in GHSR WT compared with GHSR KO mice ($F_{1,34} = 6.55$, $P < 0.05$).

Concentrations of the 5-HT metabolite 5-HIAA in the amygdala, PVN and prefrontal cortex were increased in stressed mice, regardless of their genotype ($F_{1,34} = 7.74$, $P < 0.05$ for amygdala; $F_{1,34} = 21.55$, $P < 0.001$ for PVN; $F_{1,34} = 11.47$, $P < 0.05$ for prefrontal cortex; Table 1). Interestingly, regardless of the manipulation, GHSR KO mice had lower levels of 5-HIAA in the PVN than GHSR WT mice (main effect of genotype, $F_{1,34} = 4.24$, $P < 0.05$; Fig. 6).

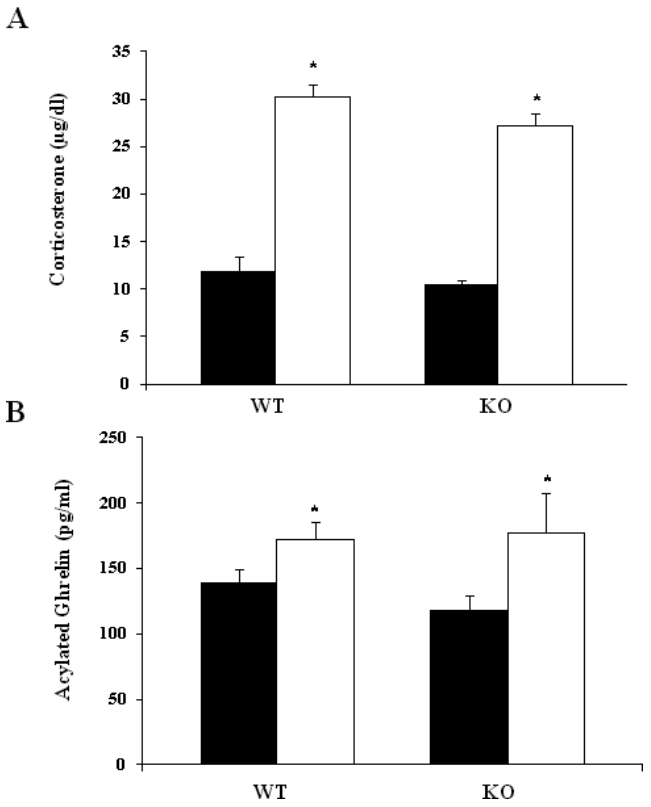


FIG. 3. Mean levels of (A) circulating corticosterone (± SEM) and (B) mean levels of circulating acylated ghrelin (± SEM) at the time of death in non-stressed (black bars) and stressed (white bars) GHSR KO and wild-type mice. *$P < 0.05$ compared with non-stressed controls ($n = 10$ mice per group).

## Discussion

Stress is an expensive energetic state, and organisms respond to this challenge by mobilizing energy that is easy to break down into substrates. Thus, in the face of acute stress, carbohydrates are preferentially metabolized, while anabolic processes are delayed. These processes have been previously linked to the peripheral actions of glucocorticoids (Sapolsky *et al.*, 1986; Black, 2006; Depke *et al.*, 2008). In the present study, stressed WT mice showed an overall decrease in their total caloric intake that was independent of the type of diet the mice consumed, energy efficiency and a reduction in body weight gain similar to that reported in the literature (Depke *et al.*, 2008). Chronically stressed GHSR KO mice, however, do not show these metabolic changes despite having physiologically relevant glucocorticoid responses to stressors. Therefore, our data suggest that ghrelin, like glucocorticoids, is important for the metabolic shift required to deal with chronic stress. Although levels of corticosterone were overall lower in GHSR KO mice, corticosterone responses to stress were not different between strains, making it unlikely that this hormone mediates the inter-strain changes in body weight, caloric intake and efficiency in response to stressors. The presence of glucocorticoids, however, may be necessary for ghrelin to maintain adiposity in WT stressed mice, as intact adrenals are necessary for the adipogenic effects of ghrelin mimetics (Tung *et al.*, 2004).

In parallel with the changes in body weight, caloric intake and efficiency in response to stressors, several monoamine alterations were observed in stressed animals, some of which differed between GHSR KO and WT mice. There were few differences in the content of NE and DA and their metabolites between non-stressed GHSR KO and WT mice. Higher levels of MHPG, however, were observed in the amygdala,

TABLE 1. Summary of changes in the content of amine and monoamine neurotransmitters and their metabolites in the brains of GHSR KO and WT mice following exposure to chronic unpredictable stressors (ean concentrations were in the order of ng/mg of tissue protein)

| | | WT control | WT stress | KO control | KO stress |
|---|---|---|---|---|---|
| NE & MHPG | | | | | |
| Amygdala | NE | 23.27 ± 1.02 | 24.42 ± 0.95 | 25.44 ± 1.33 | 23.97 ± 1.39 |
| | MHPG* | 4.84 ± 0.64 | 8.11 ± 1.81 | 4.78 ± 0.75 | 7.99 ± 1.17 |
| Arcuate nucleus | NE | 17.67 ± 1.87 | 17.91 ± 1.17 | 17.62 ± 1.55 | 19.62 ± 1.80 |
| | MHPG† | 4.41 ± 0.81 | 2.92 ± 0.53 | 3.07 ± 0.46 | 4.33 ± 0.50 |
| Hippocampus | NE | 7.16 ± 0.52 | 6.90 ± 0.71 | 8.30 ± 0.86 | 6.60 ± 0.51 |
| | MHPG | 4.65 ± 0.59 | 5.27 ± 0.45 | 4.05 ± 0.47 | 5.92 ± 0.99 |
| Locus coeruleus | NE | 16.87 ± 1.02 | 17.29 ± 1.48 | 19.13 ± 1.36 | 18.68 ± 1.40 |
| | MHPG | 3.43 ± 0.34 | 5.23 ± 1.25 | 3.71 ± 0.34 | 4.55 ± 0.72 |
| Prefrontal cortex | NE | 5.97 ± 0.31 | 6.29 ± 0.64 | 5.81 ± 0.29 | 6.60 ± 0.54 |
| | MHPG† | 3.37 ± 0.22 | 4.59 ± 0.65 | 2.99 ± 0.20 | 6.34 ± 0.74 |
| Paraventricular nucleus | NE | 39.31 ± 1.37 | 39.99 ± 0.81 | 37.36 ± 2.29 | 42.84 ± 1.65 |
| | MHPG* | 4.79 ± 0.29 | 5.51 ± 0.35 | 4.64 ± 0.31 | 5.40 ± 0.39 |
| DA, HVA & DOPAC | | | | | |
| Nucleus accumbens | DA | 97.23 ± 15.56 | 145.98 ± 22.98 | 90.13 ± 14.28 | 111.92 ± 18.37 |
| | DOPAC† | 19.19 ± 1.42 | 37.40 ± 4.50 | 26.86 ± 4.14 | 28.80 ± 3.68 |
| | HVA* | 1.87 ± 0.32 | 2.93 ± 0.50 | 1.933 ± 0.71 | 3.28 ± 0.71 |
| Prefrontal cortex | DA | 2.82 ± 0.30 | 2.51 ± 0.29 | 2.23 ± 0.21 | 2.71 ± 0.23 |
| | DOPAC* | 1.92 ± 0.20 | 2.72 ± 0.43 | 1.65 ± 0.20 | 2.21 ± 0.31 |
| | HVA‡ | 1.16 ± 0.11 | 1.76 ± 0.29 | 2.01 ± 0.57 | 2.97 ± 0.69 |
| Paraventricular nucleus | DA | 17.13 ± 0.81 | 17.75 ± 0.57 | 15.49 ± 1.18 | 17.81 ± 0.81 |
| | DOPAC | 7.02 ± 0.29 | 7.40 ± 0.23 | 6.53 ± 0.37 | 7.32 ± 0.33 |
| | HVA* | 7.77 ± 0.63 | 13.84 ± 1.26 | 7.87 ± 1.30 | 13.55 ± 1.25 |
| 5-HT & 5-HIAA | | | | | |
| Amygdala | 5-HT | 26.80 ± 2.02 | 22.76 ± 2.90 | 22.39 ± 1.75 | 26.78 ± 3.66 |
| | 5-HIAA* | 20.55 ± 1.35 | 25.59 ± 3.74 | 18.85 ± 1.34 | 27.49 ± 3.03 |
| Arcuate nucleus | 5-HT* | 14.83 ± 1.57 | 15.60 ± 1.99 | 19.42 ± 1.43 | 19.23 ± 1.48 |
| | 5-HIAA | 18.56 ± 1.91 | 21.05 ± 1.96 | 19.59 ± 2.24 | 22.02 ± 1.44 |
| Hippocampus | 5-HT | 7.03 ± 0.28 | 6.40 ± 0.65 | 6.89 ± 0.56 | 6.57 ± 0.63 |
| | 5-HIAA | 5.25 ± 0.27 | 5.78 ± 0.47 | 5.94 ± 0.29 | 6.57 ± 0.56 |
| Prefrontal cortex | 5-HT | 3.63 ± 0.26 | 3.67 ± 0.52 | 4.19 ± 0.17 | 3.88 ± 0.31 |
| | 5-HIAA* | 3.53 ± 0.25 | 4.94 ± 0.65 | 3.98 ± 0.21 | 4.93 ± 0.23 |
| Paraventricular nucleus | 5-HT | 14.48 ± 1.22 | 15.06 ± 0.96 | 14.21 ± 1.46 | 15.95 ± 1.27 |
| | 5-HIAA*,‡ | 20.27 ± 1.27 | 31.70 ± 3.30 | 18.12 ± 1.29 | 13.55 ± 1.25 |

NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; DA, dopamine; HVA, homovanillic acid; DOPAC, 3,4-dihydroxy-phenylacetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindolacetic acid. *Significant main effect of treatment ($P < 0.05$). †Significant genotype–treatment interaction ($P < 0.05$). ‡Significant main effect of genotype ($P < 0.05$).

PVN, hippocampus and prefrontal cortex, and tended to increase in the locus coeruleus of stressed mice regardless of their genotype. This effect has been previously reported, and demonstrates that the unpredictable stress paradigm was effective in producing a significant neurochemical response in the brain (Anisman & Zacharko, 1990; Shanks *et al.*, 1994; Tannenbaum *et al.*, 2002; Ulrich-Lai & Herman, 2009).

The only region where NE neurotransmission was not increased overall by the stressor was the ARC. Here, MHPG content was reduced in stressed WT mice but not in GHSR KO mice. The ARC nucleus is an important conduit for the regulation of metabolism by peripheral signals such as ghrelin and corticosterone (Abizaid & Horvath, 2008). Cells within the ARC produce orexigenic (i.e. neuropeptide Y and Agouti-related peptide), and anorectic (cocaine amphetamine related transcipt (CART), alpha-melanin stimulating hormone (α-MSH)) peptides that regulate energy balance and these peptides are modulated by NE (Wellman, 2000; Abizaid & Horvath, 2008). Indeed, NE stimulates feeding as well as the expression of neuropeptide Y and Agouti-related peptide in the ARC of rats, and ghrelin's effects on food intake depend on noradrenergic inputs to the hypothalamic ARC (Fraley *et al.*, 2002; Fraley & Ritter, 2003; Date *et al.*, 2006). Lower NE utilization in the ARC could therefore explain the decrease in general caloric intake that was observed in stressed WT mice. Of course, this is speculative and it will be necessary to determine whether the effects of the stressor on caloric intake and body weight are modifiable by NE alterations at the ARC.

Overall, GHSR KO mice tended to be slightly smaller than WT mice and to accumulate less body fat despite having access to a high-calorie diet, a phenotype reported by Pfluger *et al.* (2008). This may be explained by strain differences in serotonin content in the ARC, where serotonin levels were higher in GHSR KO mice than in WT mice regardless of treatment. Serotonin acts on pro-opiomelanocortin cells in the ARC to stimulate the release of α-MSH, a potent stimulator of energy expenditure with anorexic properties (Heisler *et al.*, 2002; Zhou *et al.*, 2005). These data would support the idea that ghrelin inhibits the release of serotonin in the ARC of mice, as shown previously (Brunetti *et al.*, 2002).

Chronic stress did increase levels of serotonin metabolites in the PVN of mice, an effect particularly evident in WT mice. Increased serotonin neurotransmission in the PVN following stress has been associated with increased production of corticotropin-releasing hormone in the PVN, and with decreased food intake and body weight (Le Feuvre *et al.*, 1991; Jorgensen *et al.*, 1998, 2002). Thus, it seems likely that both the overall decreases in caloric intake seen in stressed WT mice are related to stress-induced increases in serotonin utilization in the PVN that are not seen in GHSR KO mice.

In corroboration of previous research, NE utilization was increased in the prefrontal cortex of stressed mice (Anisman & Zacharko, 1990;

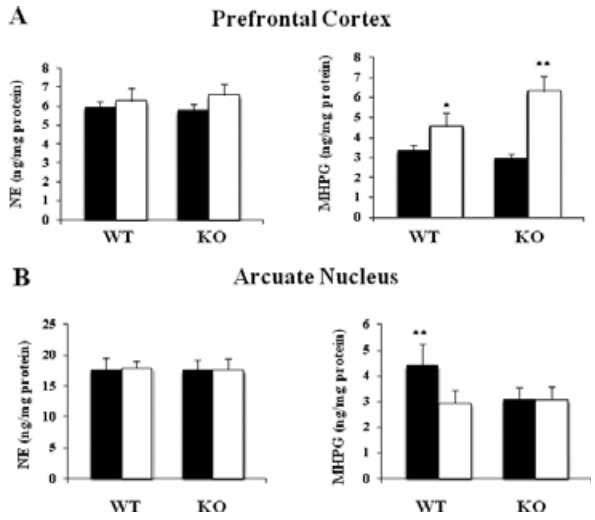


FIG. 4. Mean levels of NE (± SEM) and MHPG (± SEM) in (A) the prefrontal cortex and (B) arcuate nucleus of the hypothalamus (ARC) of non-stressed (black bars) and stressed (white bars) GHSR KO and wild-type mice. *$P < 0.05$ compared with non-stressed controls, **$P < 0.05$ compared with all other groups.

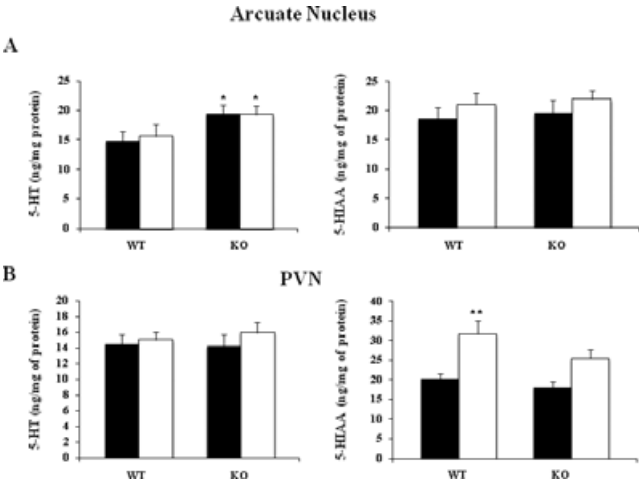


FIG. 6. Mean levels of 5-HT (± SEM) and 5-HIAA (± SEM) in (A) the arcuate nucleus (ARC) and (B) the paraventricular nucleus of the hypothalamus (PVN) of non-stressed (black bars) and stressed (white bars) GHSR KO and wild-type mice. *$P < 0.05$ compared with WT mice, **$P < 0.05$ compared with all other groups.

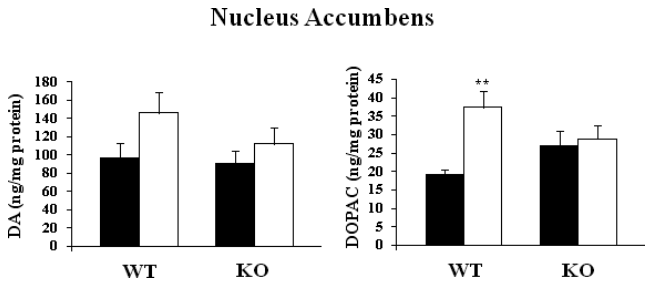


FIG. 5. Mean levels of DA (± SEM) and DOPAC (± SEM) in the nucleus accumbens of non-stressed (black bars) and stressed (white bars) GHSR KO and wild-type mice. **$P < 0.05$ compared with all other groups.

Shanks *et al.*, 1994; Birnbaum *et al.*, 1999; Arnsten, 2000, 2009; Tannenbaum *et al.*, 2002). In this instance, stressed GHSR KO mice showed greater levels of MHPG than stressed WT mice. The prefrontal cortex plays an important role in regulating HPA axis activity following acute stressors (Diorio *et al.*, 1993). Nevertheless, although increased NE neurotransmission may lower the activity of the HPA axis, excess NE activity in the prefrontal cortex has been associated with impaired stress regulation and ultimately psychopathology (Birnbaum *et al.*, 1999; Arnsten, 2000, 2009). These results might suggest that ghrelin secretion in response to stressors serves to limit NE neurotransmission in the prefrontal cortex, and that unrestrained NE release and utilization in the prefrontal cortex may be detrimental to GHSR KO mice and render them vulnerable to stressor-induced psychopathology. Indeed, Lutter *et al.* (2008) have shown that GHSR KO mice display more depressive-like behaviors following chronic exposure to a social stressor than their WT counterparts.

As shown previously (Anisman & Zacharko, 1990; Tannenbaum *et al.*, 2002), the chronic unpredictable stressor paradigm used in the present study also resulted in increased levels of DA metabolites in PVN, prefrontal cortex and nucleus accumbens. It is particularly interesting that in the nucleus accumbens, DA utilization was significantly increased only in WT mice. DA released into the nucleus accumbens comes primarily from cells in the midbrain VTA and is associated with reward-seeking behaviors (Wise, 2004). About 50–60% of the VTA DA cells contain ghrelin receptors and these cells are stimulated by ghrelin (Guan *et al.*, 1997; Abizaid *et al.*, 2006; Zigman *et al.*, 2006). Peripheral injections and local ghrelin infusions into the VTA produce DA release in mice and rats, an effect that is absent in GHSR KO mice (Abizaid *et al.*, 2006; Jiang *et al.*, 2006; Jerlhag *et al.*, 2007; Jerlhag, 2008). As such, it is possible that stress-induced increases in DA release into the nucleus accumbens are mediated in part by ghrelin acting on VTA DA neurons. This conclusion is consistent with previous observations (Abizaid *et al.*, 2006; Abizaid, 2009) and raises the possibility that ghrelin might contribute to the rewarding effects of ghrelin in appetitive situations.

Here we used mice with genetic deletion of the GHSR gene and as such developed in the absence of this receptor. This probably caused developmental patterns that are different from those of their WT littermates, and thus it is difficult to determine if the data obtained are due to abnormal developmental patterns or to the actions of ghrelin on its receptor in adult animals. Future studies using either pharmacological blockade of the receptor or genetic knockdown of the receptor in adult animals may provide for more specific answers to this questions. There is also the possibility that receptors other than GHSR bind ghrelin to alter adiposity (Halem *et al.*, 2005). However, the relative contribution of these putative receptors remains obscure.

Overall, our results indicate that ghrelin, a hormone normally associated with the regulation of appetite and energy balance, plays an important role in mediating the metabolic and neurochemical responses to stressors. In line with this perspective, several studies have shown that ghrelin is increased following acute and chronic stressors (Asakawa *et al.*, 2001; Kristenssson *et al.*, 2006; Ochi *et al.*, 2008; Zheng *et al.*, 2009). Acutely, the release of ghrelin appears to enhance the activity of the HPA axis and it may be important in the regulatory feedback mechanisms controlling this endocrine response (Asakawa *et al.*, 2001; Giordano *et al.*, 2006; Stevanovic *et al.*, 2007). Additionally, ghrelin could play a role in the allostatic changes required to respond to acute and chronic stressors. In this context, ghrelin may serve to maintain energy balance in stressed organisms by increasing the release of glucose available for utilization while

protecting fat stores from depletion (Tschop *et al.*, 2000; van der Lely, 2009). Continuous exposure to stress may, however, unmask negative metabolic consequences that could be related to ghrelin, including hyperglycemia, increased adiposity and ultimately obesity.

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## Abbreviations

5-HIAA, 5-hydroxyindolacetic acid; 5-HT, serotonin; DA, dopamine; DOPAC, 3,4-dihydroxy-phenylacetic acid; GHSR, ghrelin receptor gene; HPLC, high-performance liquid chromatography; HVA, homovanillic acid; KO, knockout; MHPG, 3-methoxy-4-hydroxyphenylglycol; NE, norepinephrine; PVN, paraventricular nucleus of the hypothalamus; RIA, radioimmunoassay; VTA/SN, ventral tegmentum and substantia nigra; WT, wild-type.

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