Ghrelin Regulates the Hypothalamic-Pituitary-Adrenal Axis and Restricts Anxiety After Acute Stress

Sarah J. Spencer, Lu Xu, Melanie A. Clarke, Moyra Lemus, Alex Reichenbach, Bram Geenen, Tamás Kozicz, and Zane B. Andrews

Background: Ghrelin plays important roles in glucose metabolism, appetite, and body weight regulation, and recent evidence suggests ghrelin prevents excessive anxiety under conditions of chronic stress.

Methods: We used ghrelin knockout (ghr−/−) mice to examine the role of endogenous ghrelin in anxious behavior and hypothalamic-pituitary-adrenal axis (HPA) responses to acute stress.

Results: Ghr−/− mice are more anxious after acute restraint stress, compared with wild-type (WT) mice, with three independent behavioral tests. Acute restraint stress exacerbated neuronal activation in the hypothalamic paraventricular nucleus and medial nucleus of the amygdala in ghr−/− mice compared with WT, and exogenous ghrelin reversed this effect. Acute stress increased neuronal activation in the centrally projecting Edinger-Westphal nucleus in WT but not ghr−/− mice. Ghr−/− mice exhibited a lower corticosterone response after stress, suggesting dysfunctional glucocorticoid negative feedback in the absence of ghrelin. We found no differences in dexamethasone-induced Fos expression between ghr−/− and WT mice, suggesting central feedback was not impaired. Adrenocorticotropic hormone replacement elevated plasma corticosterone in ghr−/−, compared with WT mice, indicating increased adrenal sensitivity. The adrenocorticotropic hormone response to acute stress was significantly reduced in ghr−/− mice, compared with control subjects. Pro-opiomelanocortin anterior pituitary cells express significant growth hormone secretagogue receptor.

Conclusions: Ghrelin reduces anxiety after acute stress by stimulating the HPA axis at the level of the anterior pituitary. A novel neuronal growth hormone secretagogue receptor circuit involving urocortin 1 neurons in the centrally projecting Edinger-Westphal nucleus promotes an appropriate stress response. Thus, ghrelin regulates acute stress and offers potential therapeutic efficacy in human mood and stress disorders.

Key Words: Anxiety, depression, ghrelin, hypothalamic-pituitary-adrenal axis, knockout, stress

Ghrelin is an important gastrointestinal peptide that regulates feeding and metabolism. Recent findings suggest ghrelin also has a critical role in integrating central circuitry involved in anxiety and responses to stress. In humans, the degree to which ghrelin is elevated by stress directly correlates with the magnitude of the stress response (i.e., those that have higher cortisol responses to stress produce greater increases in plasma ghrelin) (1). In addition, circulating ghrelin levels are reduced in those suffering from depression (2–3), and people with polymorphisms of the gene encoding ghrelin are more likely to suffer from depressive disorders (3–4).

In animal models, acute and chronic stress both elevate plasma ghrelin (5–9), as does stimulating the hypothalamic-pituitary-adrenal (HPA) axis with the synthetic glucocorticoid dexamethasone (10). Lutter *et al.* (11) found that increasing plasma ghrelin levels with SC ghrelin injection or caloric restriction reduces anxiety in a model of chronic social defeat in a ghrelin receptor (growth hormone secretagogue receptor; GHSR)-dependent manner, suggesting an anxiolytic role for the peptide after chronic stress. Moreover, ghrelin mediates reward sensation after chronic stress, because chronically stressed WT mice show conditioned place preference for high-fat food, which is not seen in GHSR−/− mice (12). Because chronic stress increases acyl ghrelin levels, Chuang *et al.* (12) postulated that ghrelin promotes a preference for high-fat diet as a mechanism to cope with chronic stress. Although these studies clearly illustrate GHSR signaling reduces anxiety associated with chronic stress, the effect of ghrelin on anxious behavior and the stress axis after acute stress remains unknown. Additionally, the GHSR-dependent effects on anxiety previously described (11,12) might be independent of the hormonal actions of endogenous ghrelin, because up to 50% of GHSR signaling occurs in the absence of ghrelin ligand binding (13). Therefore we used ghrelin knockout (ghr−/−) mice to investigate the hypothesis that endogenous ghrelin mediates anxious behavior and HPA axis responses after acute stress.

Methods and Materials

Animals

All experiments were conducted in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals and were approved by the Monash University School of Biomedical Sciences Animal Ethics Committee.

Ghr−/− mice (on a C57/B6 background) were obtained from Regeneron Pharmaceuticals (Tarrytown, New York) and bred in the Monash Animal Services facility. This genetic mouse line has been described previously (14,15). The GHSR-green fluorescence protein (GFP) mice were obtained from the Mouse Mutant Regional Resource Center at University of California at Davis. This mouse was generated by the Gene Expression Nervous System Atlas (GENSAT) project at Rockefeller University and contains a modified BAC in which a GFP reporter is inserted immediately upstream of the coding sequence for the GHSR gene.

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Mice were maintained at 22°C on a 12-hour light/dark cycle (7:00 AM–7:00 PM) with pelleted mouse chow and water available ad libitum. All experiments were conducted with adult male WT or ghr−/− mice of 8–10 weeks of age.

Elevated Plus Maze Test for Anxiety

At 8–10 weeks of age, $n = 5$ WT and 6 ghr−/− mice were tested for 7 min in the elevated plus maze test for anxiety in a novel environment, as described previously (16–18). Each mouse was placed in the center of the plus maze and filmed and later scored for the number of entries into and time spent in each of the open and closed arms with Ethovision software (Noldus Information Technology, Wageningen, The Netherlands). After 1 week recovery from the initial baseline test, the mice were given 15-min restraint stress (restraint in a ventilated Perspex tube, 3 cm in diameter, with an adjustable restraining length to a maximum of 10 cm) and tested again immediately after stress.

Open Field Test for Anxiety and Locomotor Activity

A separate group of mice ($n = 8$ WT and 14 ghr−/−) was also tested in the open field test for anxiety and locomotor activity as described previously (16,17) with modifications for mice. Each animal was placed in the center of the arena and filmed and later scored for locomotion (distance travelled), number of entries into the middle of the arena, and frequency of grooming bouts, in a period of 10 min. As with the elevated plus maze, the mice underwent a basal trial, followed 7 days later by a stress trial.

Light Dark Box Test for Anxiety

After 7-day recovery from the open field tests, the mice ($n = 10$ WT and 19 ghr−/−) were then tested in the light/dark box test for anxiety (19). Each mouse was placed in an enclosed (dark) arena (22 × 30 × 25 cm), filmed, and later scored for the time spent exploring the high light arena (22 × 30 × 25 cm) in a 5-min trial. The mice underwent a basal trial, followed 7 days later by a stress trial as described in the preceding text.

Neuronal Activation in Response to Stress

On the day of experimentation, the mice ($n = 4$–7/group) were brought into the testing room at 7:00 AM and allowed 2 hours to acclimatize to the room before 15-min restraint stress (or no stress for control subjects). Two hours after stress onset, mice were anaesthetized and perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS (4°C, pH 7.4). Brains were removed and post-fixed for 24 hours in the same fixative before being cryoprotected with 20% sucrose in PBS (4°C). Forebrains were subsequently cut with a cryostat into 40-μm coronal sections.

Neuronal activation was assessed on the basis of positive Fos-immunoreactivity (24 hours, 4°C; 1:10,000; rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, California). Fos-positive cells were counted blind in regions of interest (20,21).

Neuronal Activation in Response to Dexamethasone

We used a separate group of mice ($n = 8$/group) to assess paraventricular nucleus of the hypothalamus (PVN) neuronal activation in response to the synthetic glucocorticoid dexamethasone. Mice were given 30 μg/kg SC dexamethasone or saline and perfused 120 min later.

Neuronal Activation in Response to Stress After Ghrelin Replacement

We took a separate cohort of mice ($n = 3$–8/group) and gave them a single SC injection of ghrelin (ghrelin 1 mg/kg in saline) or vehicle. Thirty minutes after injection we gave a subset of the mice 15-min restraint stress. The mice were perfused 120 min after stress onset as described in the preceding text.

Corticosterone and Adrenocorticotropic Hormone Assay

To assess plasma hormone responses to stress or adrenocorticotropic hormone (ACTH), we decapitated mice ($n = 8$/group/time point) before or 15, 30, and 60 min after the onset of stress or 30 min after injection with 1.5 μg/kg SC ACTH or saline ($n = 8$/group). Blood was collected on ice and centrifuged, and plasma was kept at −80°C until ready for use. A standard corticosterone enzyme immunoassay kit (Abnova, Taipei, Taiwan) was used to assess plasma corticosterone, and a standard ACTH radioimmunoassay kit (Phoenix Pharmaceuticals, Burlingame, California) was used for plasma ACTH. The inter- and intra-assay variabilities for these assays were <9% coefficient of variation (CV), and lower limits of detection were 40 pg/mL and 34 pg/mL, respectively. Samples were assayed in duplicate.

Mineralocorticoid Receptor and Glucocorticoid Receptor Gene Expression

Hypothalami were quickly dissected, snap frozen in liquid nitrogen, and stored at −80°C until use. The RNA was isolated with QIAzol and an RNeasy purification kit (QIAGEN, Valencia, California). The RNA (1 μg) was transcribed to complementary DNA with an iScript cDNA synthesis kit; (Bio-Rad Laboratories, Hercules, California), following the instructions of the manufacturers. We performed real-time reverse transcription polymerase chain reaction with Taqman Gene Expression Assays (Applied Biosystems, Mulgrave, Victoria, Australia). We measured fold differences in target messenger RNA (mRNA) expression with the δ-cycle threshold method by comparison with the housekeeping gene, 18S (22,23), and expressed as mRNA relative fold change (fold increase) as described previously (21,24,25).

Data Analysis

Each parameter of the behavioral tests was compared between WT and ghr−/− mice with a repeated measures analysis of variance with genotype as the between factor and no-stress/stress as the repeated measure. We also analyzed the change in behaviors between basal and stressed conditions; Student unpaired *t* tests. Two-way analyses of variance were used for all other statistical analyses with Student-Neumann-Keuls post hoc comparisons as appropriate. Data are presented as the mean ± SEM. Statistical significance was assumed when $p < .05$.

Results

Genetic Ablation of Ghrelin Enhances the Anxiogenic Effect of Acute Restraint Stress

To determine the effects of endogenous ghrelin on anxiety, we tested WT and ghr−/− mice under basal conditions and after 15-min acute restraint stress in the elevated plus maze, open field, and light/dark box tests for activity and anxiety.

Elevated Plus Maze. The total number of open arm entries did not differ between ghr−/− and WT mice under basal or stressed conditions. However, the difference between basal and post-stress trials clearly demonstrated a significant increase in open arm exploration in WT mice that is not seen in ghr−/− (Figures 1A and 1B). Under basal conditions, ghr −/− mice spent more time in the open arms compared with WT (Figure 1C). However, acute restraint stress also reduced the time spent in the open arms more in ghr−/− mice than in WT, when the differences between basal and post-stressed trials were compared (Figure 1D). The percentage of open arm entries relative to total entries did not differ between genotypes,

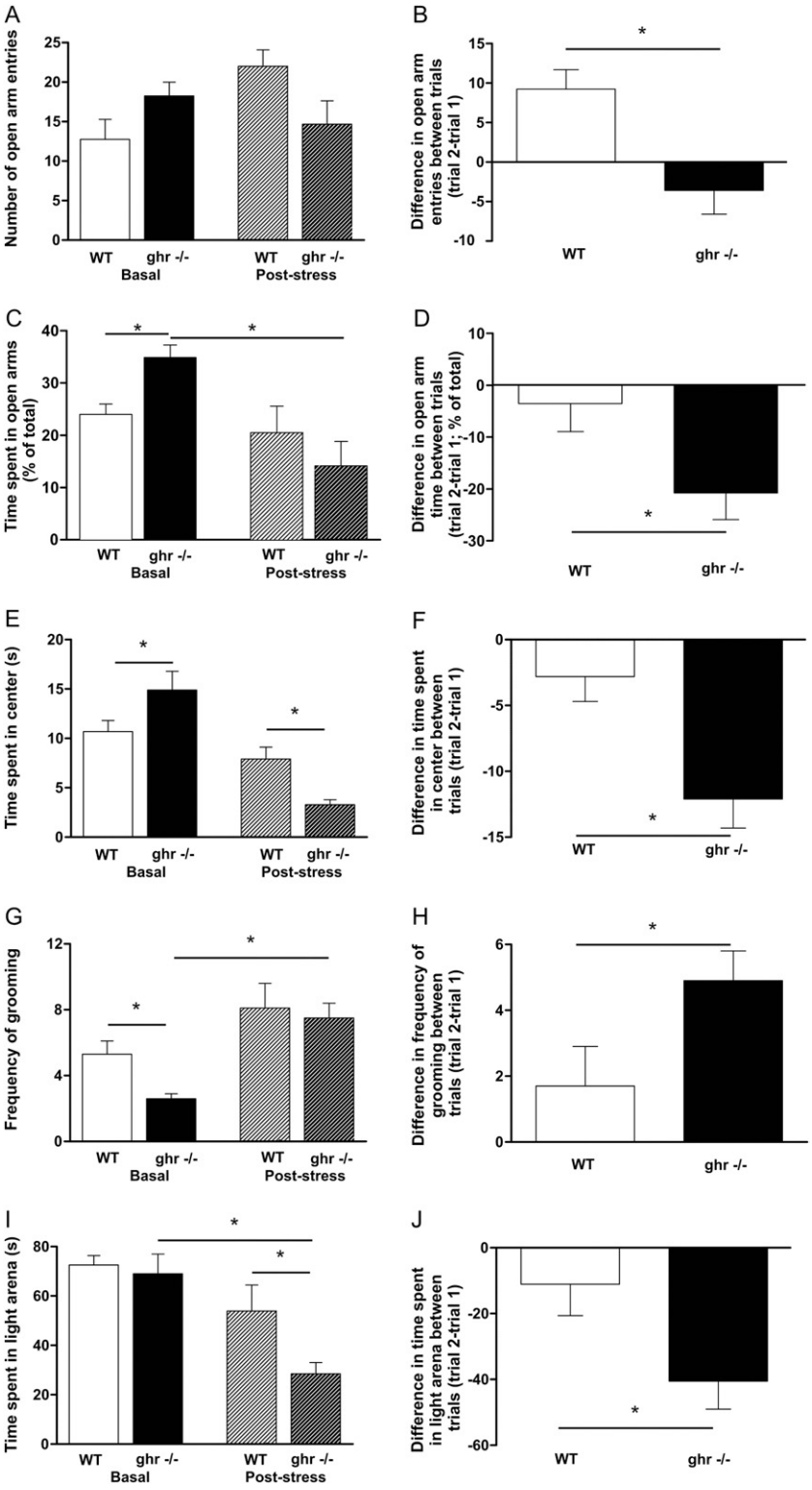

Figure 1. Genetic ablation of ghrelin enhances the anxiogenic effect of stress in the elevated plus maze, open field, and light-dark box. **(A)** Number of entries into the open arms of the elevated plus maze before (basal) and after 15-min restraint stress [significant stress × genotype interaction $F(3,18) = 6.98$, $p = .017$]. **(B)** Difference between basal and post-stress trials in number of entries into the open arms [$t(9) = 3.22$, $p = .011$]. **(C)** Time spent in open arms [significant stress × genotype interaction $F(3,18) = 5.15$, $p = .036$; Student-Neumann-Keuls (SNK) $p < .05$]. **(D)** Difference between basal and post-stress trials in time spent in open arms [$t(9) = 2.87$, $p = .019$]. **(E)** Time spent in the center of the open field arena before (basal) and after 15-min restraint stress [significant stress × genotype interaction $F(3,40) = 9.22$, $p = .004$; SNK $p < .05$]. **(F)** Difference between basal and post-stress trials in the time spent in the center [$t(20) = 2.85$, $p = .01$]. **(G)** Frequency of grooming in the open field arena [significant effect of stress $F(3,40) = 19.30$, $p < .001$; SNK $p < .05$]. **(H)** Difference between basal and post-stress trials in the frequency of grooming in the open field arena [$t(20) = 2.14$, $p = .045$]. **(I)** Time spent in the light arena of the light-dark box before (basal) and after 15-min restraint stress [significant effect of stress $F(3,60) = 16.64$, $p < .001$; SNK $p < .05$]. **(J)** Difference between basal and post-stress trials in the time spent in the light arena [$t(28) = 2.30$, $p = .028$]. $N = 10–19$ mice/group. *$p < .05$. Data are mean + SEM. ghr−/−, ghrelin knockout mice; WT, wild-type mice.

indicating the absence of ghrelin did not affect total movement in the maze (data not shown).

Open Field. The ghr−/− mice exhibited more anxiety after stress in the open field test for activity and anxiety, as with the elevated plus maze. Under nonstressed conditions, ghr−/− mice spent more time exploring the center of the open field arena than WT. However, acute restraint stress reversed this effect and significantly reduced center time in ghr−/− mice compared with WT (Figure 1E). The differences between the trials confirmed that acute restraint stress significantly reduced center time in ghr−/− mice compared with WT (Figure 1F). Grooming in novel environments also indicates anxiety (26), and ghr−/− mice groomed less in the basal trial than WT mice (Figure 1G). However, acute restraint stress abolished this difference, and inter-trial analysis demon-

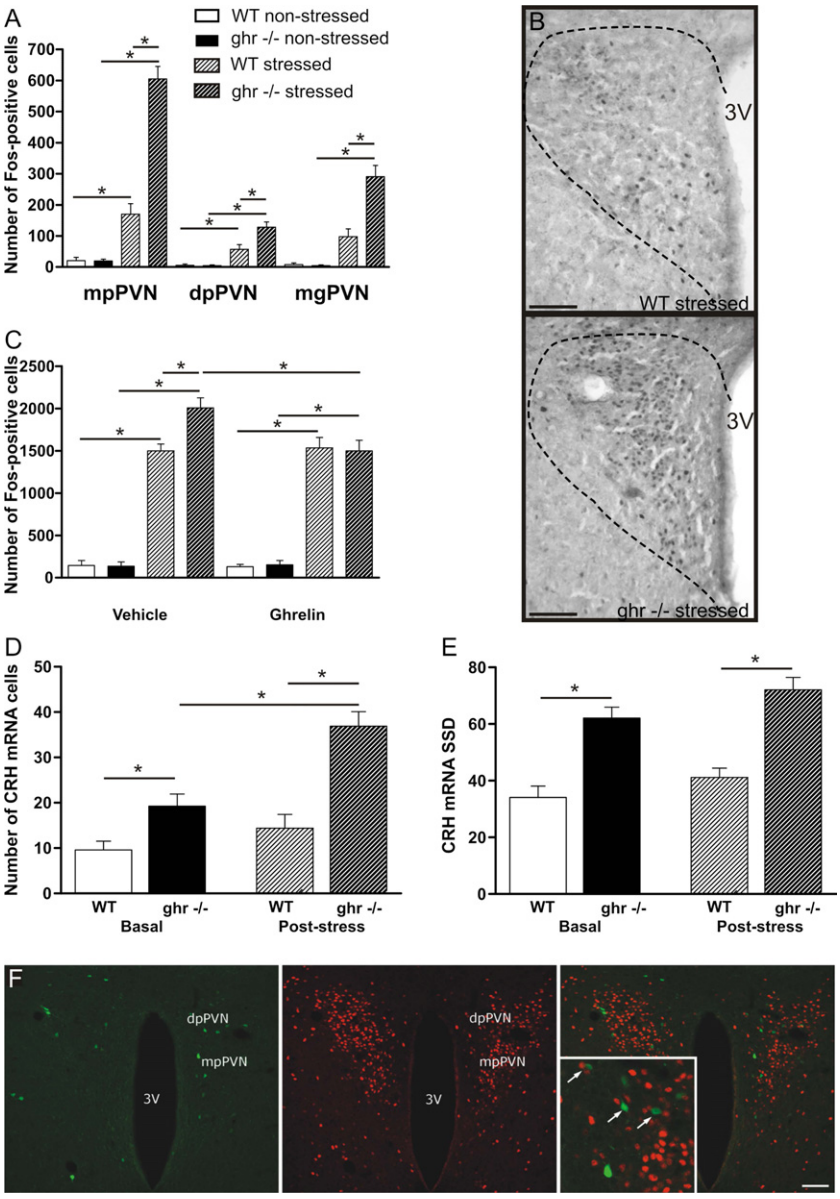

Figure 2. Genetic ablation of ghrelin exacerbates central responses to stress. **(A)** Neuronal activation after restraint stress in the medial parvocellular (mp) [significant stress × genotype interaction $F(3,20) = 44.2$, $p < .001$; Student-Neumann-Keuls (SNK) $p < .05$], dorsal parvocellular (dp) [significant stress × genotype interaction $F(3,20) = 6.6$, $p = .02$; SNK $p < .05$], and magnocellular (mg) [significant stress × genotype interaction $F(3,20) = 12$, $p = .003$; SNK $p < .05$] paraventricular nucleus of the hypothalamus (PVN), as assessed by numbers of Fos-immunoreactive cells. **(B)** Representative photomicrograph of the neuronal activation in the PVN after stress in a wild-type (WT) mouse and a ghrelin knockout (ghr−/−) mouse. **(C)** Neuronal activation after restraint stress in the PVN in the presence of replacement ghrelin (or vehicle) as assessed by numbers of Fos-immunoreactive cells [significant stress effect $F(7,37) = 258.9$, $p < .001$; SNK $p < .05$]. **(D)** Number of cells in the PVN expressing corticotropin-releasing hormone (CRH) messenger RNA (mRNA) [significant stress × genotype interaction $F(3,19) = 4.68$, $p = .043$; SNK $p < .05$]. **(E)** PVN CRH hybridization strength [specific signal density; significant effect of genotype $F(3,19) = 47.51$, $p < .001$; SNK $p < .05$]). **(F)** Representative photomicrograph of growth hormone secretagogue receptor-green fluorescence protein cells (green), Fos-immunoreactive cells (red), and an overlay illustrating the absence of activated GHSR-GFP cells (which would be illustrated in yellow) in the PVN after stress. Scale bars = 100 μm. dp dorsal parvocellular; mp, medial parvocellular; mg magnocellular; 3V, third ventricle; WT, wild-type mice.

strated that ghr−/− mice groom significantly more compared with WT mice after restraint (Figure 1H). Stress reduced the total distance travelled in both genotypes, but there were no differences in open field locomotor activity between the groups (data not shown).

Light/Dark Box. We detected no difference between genotypes under basal conditions (Figure 1I), unlike in the elevated plus maze and the open field tests. However, acute restraint stress significantly reduced time spent in the light arena in ghr−/− mice compared with WT mice, and the inter-trial analysis confirmed that ghr−/− mice spent less time exploring the light arena after stress, consistent with the other tests (Figure 1J).

Taken together, our results from three independent anxiety tests clearly demonstrate that ghrelin plays an important role in responses to acute stress. Intriguingly, results from the plus maze and open field tests suggest ghrelin actually elevates anxiety under basal conditions and reduces it under stressed conditions, highlighting a novel dual role for ghrelin in anxiety behavior.

Genetic Ablation of Ghrelin Exacerbates Central Responses to Stress

Acute stress increased neuronal activation (numbers of Fos-immunoreactive cells) in the medial parvocellular, dorsal parvocellular, and magnocellular regions of the PVN in both genotypes as expected. However, Fos activation in the PVN was significantly elevated in ghr−/− relative to WT mice (Figures 2A and 2B). Ghrelin replacement with a single injection of 1 mg/kg SC ghrelin reversed this effect in ghr−/− mice and had no impact on the WT (Figure 2C). Corticotrophin-releasing hormone (CRH) is a key PVN stress peptide that stimulates ACTH synthesis and secretion from the anterior pituitary. With in situ hybridization, we observed that ghr−/− mice had more PVN CRH cells than WT in nonstressed conditions. Similar to the profile of Fos expression, acute stress significantly increased numbers of CRH-expressing cells in the PVN in ghr−/− mice relative to WT (Figures 2D and 2E). With a novel GHSR-GFP reporter mouse that expresses GFP under the control of the GHSR promoter, we found only a negligible number of GHSR-GFP neurons in medial

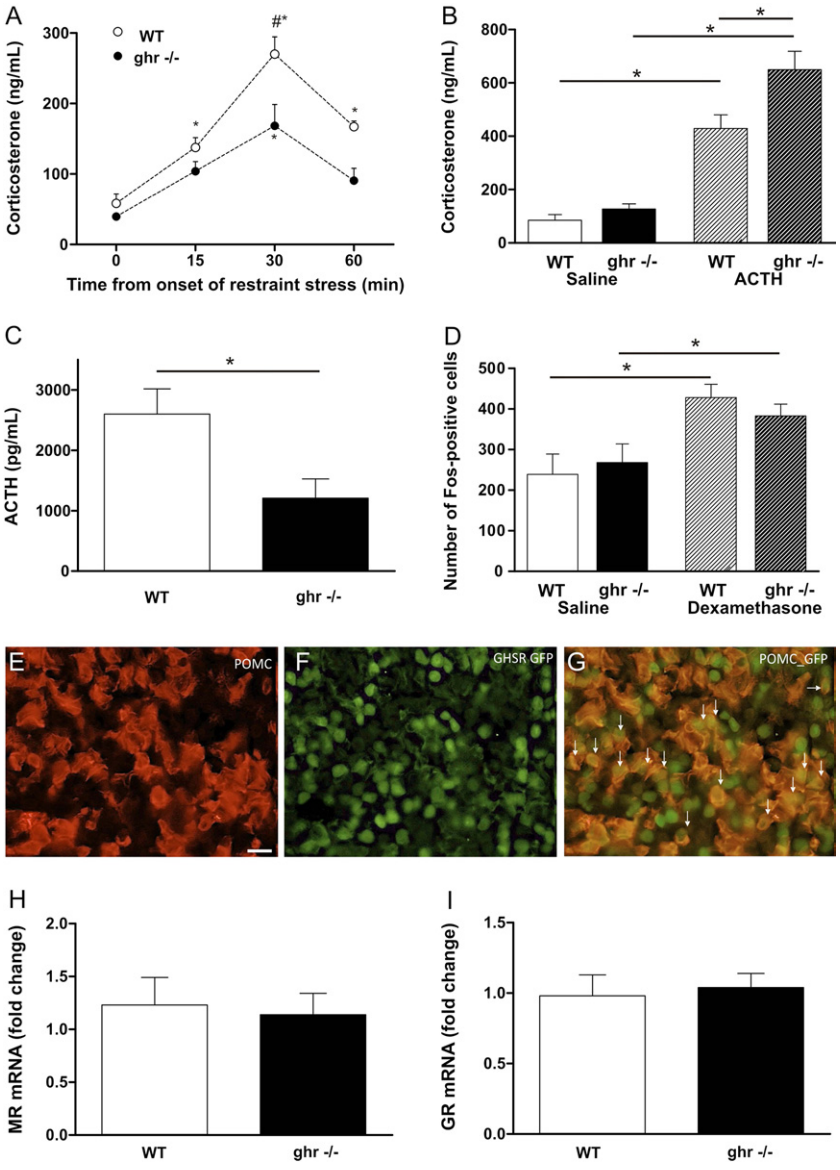

Figure 3. Genetic ablation of ghrelin attenuates adrenocorticotropic hormone (ACTH) release and subsequent glucocorticoid negative feedback in response to stress. **(A)** Corticosterone response to 15-min restraint stress [commenced at time 0; significant effect of genotype $F(7,34) = 15.11$, $p < .001$, and time (stress) $F(7,34) = 21.47$, $p < .001$; SNK $p < .05$; *significantly different from basal (time 0); #significantly different from WT]. **(B)** Plasma corticosterone concentrations in WT and ghr−/− mice 30 min after saline or 1.5 μg/kg SC ACTH injection [significant effect of genotype $F(3,28) = 8.42$, $p = .007$, and stress $F(3,28) = 91.26$, $p < .001$; *SNK $p < .05$]. **(C)** ACTH response to 15-min restraint stress [30 min after stress onset; *$t(16) = 2.63$, $p = .018$]. **(D)** Neuronal activation in the PVN of WT and ghr−/− mice after 30 μg/kg SC dexamethasone injection, as assessed by numbers of Fos-immunoreactive cells [significant stress × genotype interaction $F(3,25) = 16.42$, $p < .001$; *SNK $p < .05$]. **(E)** Proopiomelanocortin (POMC)-positive cells in the anterior pituitary. **(F)** The GHSR-positive cells in the anterior pituitary. **(G)** The POMC cells in the anterior pituitary co-expressing GHSR, as shown by white arrows. **(H)** Hypothalamic mineralocorticoid receptor (MR) mRNA. **(I)** Hypothalamic glucocorticoid receptor (GR) mRNA. Scale bars = 20 μm. n = 7–9 mice/group. *$p < .05$. Data are means + SEM. GFP, green fluorescent protein; other abbreviations as in Figure 2.

parvocellular PVN, dorsal parvocellular PVN, and magnocellular PVN (Figure 2F), indicating the PVN is unlikely to be under the direct control of ghrelin.

Genetic Ablation of Ghrelin Attenuates the Corticosterone Response to Acute Stress

Because we observed elevated stress-induced PVN activation in ghr−/− mice, we hypothesized these mice would also exhibit an increase in the plasma corticosterone response to acute stress. Unexpectedly, ghr−/− mice had lower plasma corticosterone levels at 30 min after stress than WT (Figure 3A), indicating the corticosterone response to stress is impaired in ghr−/− mice.

To determine whether reduced adrenal responsiveness to ACTH caused lower plasma corticosterone responses to stress in ghr−/− mice, we treated both groups with saline or ACTH and measured plasma corticosterone 30 min later. Intriguingly, ACTH actually increased corticosterone concentrations in ghr−/− mice relative to ACTH-treated WT mice (Figure 3B). These results show that the adrenals are supersensitive to ACTH stimulation in ghr−/− mice and point toward defective ACTH secretion from the pituitary after acute restraint stress.

In light of these findings, we measured ACTH in WT and ghr−/− mice 30 min after restraint stress and observed significantly smaller increases in plasma ACTH in ghr−/− compared with WT mice (Figure 3C). We also demonstrated that ACTH-secreting corticotropes in the pituitary gland express the GHSR, suggesting a direct functional effect of ghrelin on ACTH release in the pituitary (Figures 3E–3G).

To determine whether ghr−/− mice also display impaired corticosterone negative feedback in the CNS, we injected the glucocorticoid mimetic dexamethasone into ghr−/− and WT mice and measured Fos-immunoreactivity in the PVN. There was no significant difference between WT and ghr−/− mice in numbers of Fos-immunoreactive neurons, suggesting the central responses to glucocorticoid negative feedback are not impaired in ghr−/− mice relative to WT mice (Figure 3D). There were also no significant differences in glucocorticoid or mineralocorticoid receptor expression between WT and ghr−/− mice in the hypothalamus (Figures 3H and 3I).

Collectively, these results demonstrate that ghr−/− mice have significantly altered responses to acute stress. Although the brain has the capacity to respond to corticosterone negative feedback, less ACTH is produced from the pituitary for a given stressor, resulting in less corticosterone secretion from the adrenals. Glucocorticoid negative feedback is therefore diminished, and the PVN response exacerbated as a consequence.

Role of Ghrelin in Stress Involves the Centrally Projecting Edinger-Westphal Nucleus

To investigate the role of ghrelin in central control of anxiety and stress, we also examined a number of other brain regions that modulate the HPA axis response to stress and/or respond to ghrelin, including the amygdala, hippocampus, hypothalamic arcuate nucleus, and centrally projecting Edinger-Westphal nucleus (EWcp) (Table S1 in Supplement 1). Stress enhanced numbers of Fos-immunoreactive cells in all regions examined. However, we only observed a significant difference between genotypes in the MeA and EWcp. The MeA is a significant anxiety-processing region (27,28), and ghrelin injection directly into the MeA produces anxiogenic effects (29). In this region, the absence of ghrelin significantly enhanced the Fos response to restraint stress compared with the response seen in WT mice (Figures 4A–4C). Our studies with the GHSR-GFP reporter mouse revealed a substantial population of GHSR-GFP neurons in the MeA (Figure 4D). However, these neurons were devoid of Fos immunoreactivity, suggesting that acute stress recruited a non-GHSR neuronal population in the MeA.

The EWcp expresses high levels of GHSR mRNA (30) and houses an abundant population of urocortin 1 neurons that are regulated by restraint stress (31). With our GHSR-GFP reporter mouse, we first established that urocortin 1 neurons of the EWcp express GFP under the control of the GHSR promoter, with more than 90% of GHSR-GFP neurons also expressing urocortin 1. Under basal conditions, ghr−/− mice had significantly more Fos-positive neurons and more urocortin 1/Fos-positive neurons than WT in the EWcp (Figures 4E and 4F). Acute stress activated urocortin-1 neurons in the EWcp of WT but not ghr−/− mice. Acute stress-induced neuronal activation was seen in urocortin 1-positive GHSR-GFP neurons, the majority of the GHSR-GFP neurons being recruited (78.9 ± 3.9% in stressed vs. 8.2 ± 1.3 in control subjects) (Figures 4G and 4H). These studies provide direct neuroanatomical evidence for a functional interaction between ghrelin, the GHSR, and urocortin 1 neurons and suggest ghrelin activates urocortin 1 neurons in the EWcp to maintain appropriate HPA activation in response to acute restraint stress.

Discussion

We present the first evidence that endogenous ghrelin modulates the response to acute stress, attenuating anxious behavior and HPA axis activation under stressed conditions. In the absence of ghrelin, mice are more anxious after acute stress and have enhanced PVN activation compared with WT. Exogenous ghrelin ameliorates this central stress response. Earlier studies suggested exogenous ghrelin alters anxiety behavior, with either intracerebroventricular or intraperitoneal ghrelin increasing anxiety-like behavior in the elevated plus maze 10 min after injection (9,32). Conversely, Lutter *et al.* (11) found SC ghrelin injection or calorie restriction reduces anxiety-like behaviors. In this case, the behavioral tests were conducted 45 min after ghrelin administration or with chronically elevated ghrelin by calorie restriction. A potential confounding variable in the investigations described in the preceding text is the exogenous injection of ghrelin, because it produces a nonphysiological bolus of ghrelin. To circumvent this variable, we used a ghr−/− mouse model to investigate the physiological relevance of endogenous ghrelin in stress and anxiety. This is the first study to reveal that endogenous ghrelin prevents a hyperactive, over-anxious response to acute stress.

With these studies we have uncovered that ghrelin plays a dual role in anxious behavior (i.e., having differential effects under basal conditions and after stress). Previous studies support a dual role for ghrelin in anxious behavior, because ghrelin can be anxiogenic (9,32) or anxiolytic (11), depending upon experimental paradigms. These studies however did not examine responses to ghrelin under acute stress conditions. Moreover, acute ghrelin injection immediately encourages food-seeking behavior, which might explain the apparent anxiogenic responses.

Under nonstressed basal conditions ghr−/− mice were significantly less anxious and spent more time in the open arms of the elevated plus maze, more time in the center of the open field, and less time grooming in the open field than WT mice. Only under conditions of acute stress did ghr−/− mice manifest a more anxious profile. We used multiple behavioral tests in the current studies to highlight the robustness of these findings. These results suggest ghrelin is mildly anxiogenic during nonstressed conditions and anxiolytic after exposure to stress. Indeed, acute or chronic stress increases plasma ghrelin (5–9), suggesting this increase is required to prevent excessive anxiety. Of note, WT mice do not display an overtly anxious phenotype in the elevated plus maze or open field test; rather they show relative anxious behavior under nonstressed conditions compared with ghr−/− mice.

In the current investigation, we observed reduced plasma corticosterone, despite an enhanced PVN neuronal activation after acute stress in ghr−/− mice relative to WT. In support of this, chronic stress also reduces the corticosterone response in GHSR-null mice (12), and ghrelin-induced activation of CRH and AVP in the PVN increases plasma ACTH (33) and plasma corticosterone (9). The corticosterone response to exogenous ACTH was not affected in ghr−/− mice compared with WT, indicating ghrelin deletion did not compromise glucocorticoid production in the adrenal glands. Furthermore, the central response to the synthetic glucocorticoid dexamethasone and hypothalamic expression of glucocorticoid receptor and mineralocorticoid receptor was not different between ghr−/− and WT mice, suggesting that the central ability to respond to glucocorticoids was normal. However, in response to stress, plasma ACTH in ghr−/− mice was significantly lower than WT mice, despite the elevated PVN neuronal activation. We also observed a significant proportion of ACTH-producing cells that express the GHSR in the anterior pituitary. We therefore suggest that the lack of GHSR activation in ghr−/− mice is responsible for reduced plasma ACTH (Figure 5). These findings support the description that GHSR−/− mice have attenuated corticosterone in response to stress compared with WT (12).

GHSR-GFP expression was absent in all subdivisions of PVN (this study), suggesting that ghrelin does not have a direct effect on PVN functioning during stress, consistent with very low to nonexistent expression of GHSR mRNA in the PVN. This finding suggests that, in response to acute stress, ghrelin controls and fine-tunes HPA axis function through other important brain regions. Therefore, we examined neuronal activation in response to stress in ghr−/− and WT mice throughout the brain. Of the stress-sensitive brain regions, only the MeA and EWcp were differentially affected by the absence of ghrelin after acute stress. The MeA is closely associated with anxiety and the HPA axis response to psychological stress, acting to drive the response (27,28). Ghrelin injection directly into the MeA elicits anxiogenic effects (29). Indeed, we observed an increased neuronal activation in the MeA in ghr−/−, relative to WT mice, after stress, suggesting the MeA might underlie anxious behavior in the

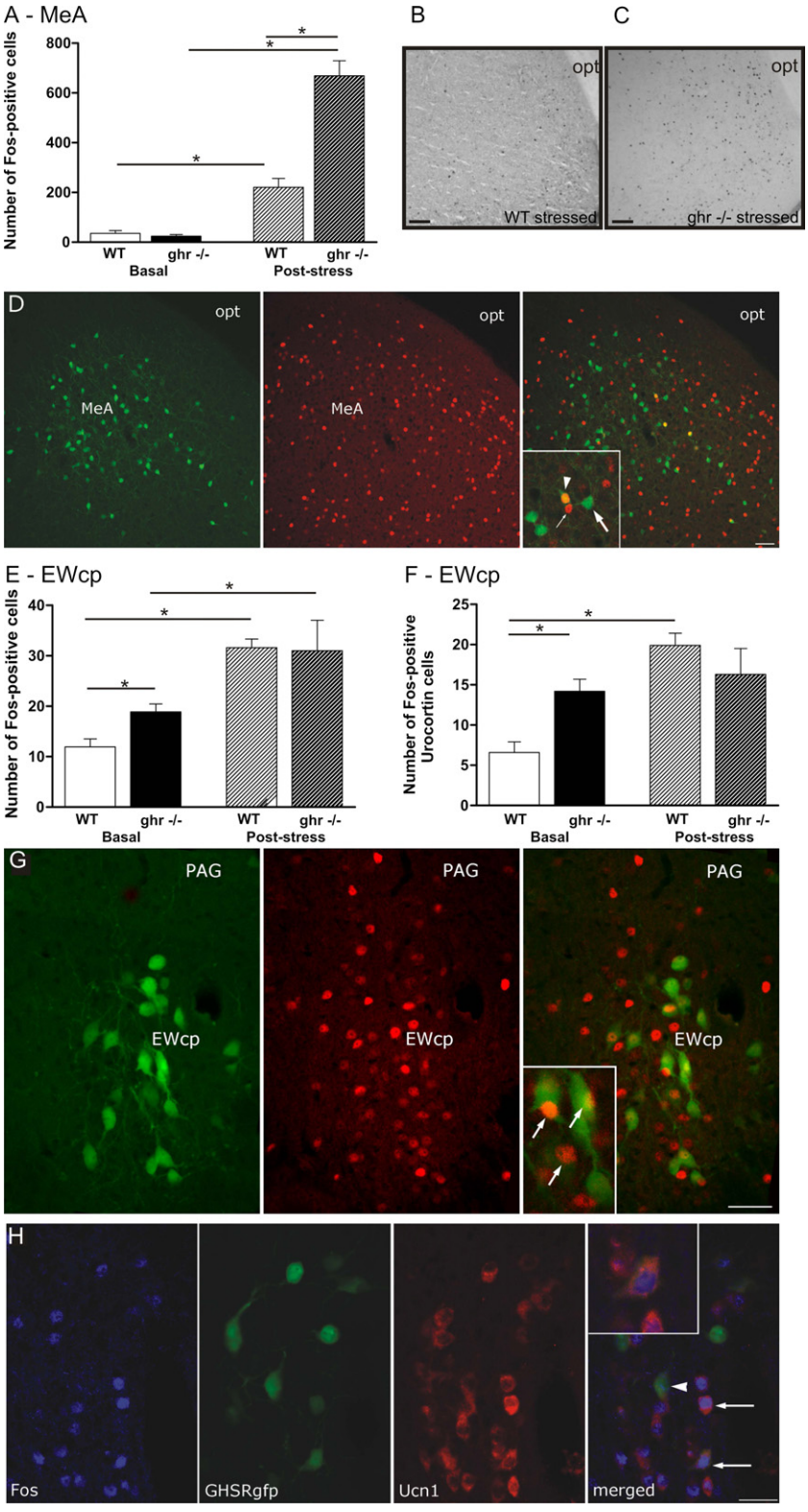

Figure 4. Genetic ablation of ghrelin alters medial amygdala (MeA) and centrally projecting Edinger Westphal (EWcp) responses to stress. **(A)** Numbers of Fos-immunoreactive cells in the MeA indicate that ghrelin restricts MeA activity after acute stress [significant stress × genotype interaction $F(3,20) = 30.2$, $p < .001$; SNK $p < .05$]. **(B)** Representative photomicrograph of the neuronal activation in the MeA after stress in a WT mouse. **(C)** Representative photomicrograph of the neuronal activation in the MeA after stress in a ghr−/− mouse. **(D)** Representative photomicrograph of GHSR-GFP cells (green), Fos-immunoreactive cells (red), and activated GHSR-GFP cells (yellow) in the MeA after stress. **(E)** Numbers of Fos-immunoreactive cells in the EWcp [significant effect of stress $F(3,18) = 18.94$, $p < .001$; SNK $p < .05$]. **(F)** Numbers of Fos-positive urocortin (Ucn1) cells in the EWcp indicate that ghrelin normally activates Ucn1 cells in the EWcp to maintain an appropriate response to acute stress [significant stress × genotype interaction $F(3,18) = 6.35$, $p = .021$; SNK $p < .05$]. **(G)** Representative photomicrograph of GHSR-GFP cells (green), Ucn1 cells (red), and GHSR-GFP Ucn1 cells (arrows) in the EWcp after stress. **(H)** Representative photomicrograph of triple-labeled Fos-immunoreactive (blue), GHSR-GFP (tan), Ucn1 (purple) cells, and activated Ucn1-GHSR-GFP cells (arrows) in the EWcp after stress (arrowhead indicates Ucn1-GHSR cell that is not Fos-positive). Scale bars = 50 μm. $n = 4–7$ mice/group. *$p < .05$. Data are means + SEM. opt, optic tract; PAG, periaqueductal grey; other abbreviations as in Figure 2.

ghr−/−. Thus, enhanced activity of the MeA after stress serves to increase the excitatory drive to the PVN in ghr−/− mice. Although the MeA contained a substantial population of GHSR-GFP neurons, these neurons were not recruited by stress. Our observations suggest that ghrelin's control over the stress response is indirect, probably via neuronal projections from other GHSR-expressing brain areas.

We ascribe a role for the EWcp in the amelioration of anxiety and stress by ghrelin. The EWcp contains some of the highest expression of GHSR in the brain (30), but no role for ghrelin has been

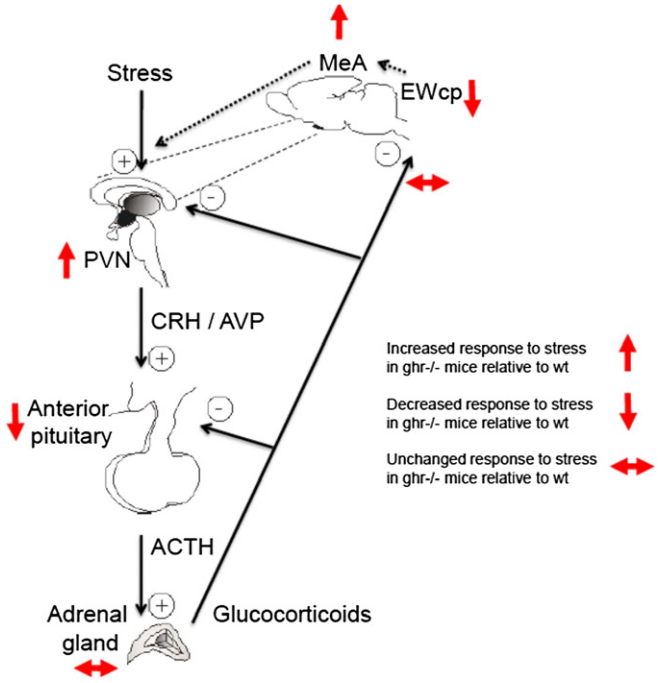

Figure 5. When an organism experiences stress, the medial PVN is activated, leading to the release of CRH and arginine vasopressin (AVP) into the anterior pituitary. Adrenocorticotropic hormone is then released into the blood stream and acts at the adrenal cortex to stimulate glucocorticoid (GC) release into circulation. The GC acts at GC receptors (GR) and mineralocorticoid receptors (MR) in the hypothalamus and elsewhere, stimulating inhibitory pathways to the PVN to prevent further activation. Our findings indicate that, in the absence of ghrelin (ghr−/− mice, red arrows), GC release from the adrenals is normal (i.e., is not attenuated in response to a standard dose of ACTH) and central responses to GC are normal (i.e., GR and MR expression in the hypothalamus is not affected, and synthetic GC induces a normal PVN Fos response). However, ACTH release from the anterior pituitary is reduced after acute stress in ghr−/− mice, leading to less corticosterone release from the adrenals and less inhibitory feedback onto the PVN. This disruption of negative feedback exacerbates PVN neuronal activation after stress in the absence of ghrelin. Ghrelin also acts at the EWcp to inhibit MeA, thus dampening the excitatory influence of the MeA on the PVN. Abbreviations as in Figures 2–4.

described until now. Here we show stress activates GHSR-expressing urocortin 1 cells in the EWcp. Various acute stressors activate urocortin-1 in the EWcp (31,34–38), and EWcp neurons remain active and express elevated urocortin 1 mRNA up to 16 hours after acute stress (37,39,40). This profile is in contrast to the relatively short-lasting (2–4 hours) activity of PVN neurons and PVN-CRH mRNA (37,39,41). These observations suggest that the EWcp plays a crucial role in maintaining homeostatic equilibrium after acute stress and that this region is involved in the later adaptive phase of the stress response. Thus EWcp neurons promote successful neuroendocrine (terminating HPA axis activation) and behavioral (anxiolysis) adaptation to acute stress (42–44). We argue the increased basal activity of EWcp in ghr−/− mice mechanistically underpins the anxiolytic phenotype of ghr−/− mice under basal conditions. Indeed, intracerebroventricular administration of benzodiazepines and selective agonists of the metabotropic glutamate receptors results in significant EWcp neuronal activation (45–47), suggesting the action of various anxiolytic drugs increases EWcp neuronal activity. It is noteworthy that the MeA contains urocortin 1-immunopositive axon terminals (48,49), raising the possibility that urocortin 1 projections from the EWcp to the MeA play a role in ghrelin-dependent regulation of MeA neurons.

We illustrate a key role for endogenous ghrelin in modulating the HPA axis and anxiety levels in response to acute stress. We demonstrate that ghrelin targets the GHSR to stimulate ACTH release from the anterior pituitary and coordinates central input to the HPA axis from the MeA and EWcp. These findings also identify a dual role for ghrelin in anxious behavior, because ghrelin increases anxiety under non-stressed conditions but decreases anxiety after acute stress. Ghrelin promotes the drive for food intake and maintains blood glucose during negative energy balance (3,50–52) as well as subserves the rewarding nature of food (12,53). We postulate that, under conditions of acute stress, ghrelin limits excessive anxious behavior by promoting the feeling of reward to ensure appropriate food-seeking behavior and maintain energy homeostasis (12). Consistent with this idea, elevated ghrelin during calorie restriction produces anxiolytic responses in the elevated plus maze (11). We hypothesize that ghrelin suppresses anxiety under acutely stressful conditions to maintain appropriate energy homeostasis. Indeed, the importance of ghrelin in controlling stress-induced anxiety might manifest only during conditions of elevated plasma ghrelin, such as negative energy balance and calorie restriction (11,54). This phenomenon represents an important evolutionary adaptation that maintains food-seeking behavior in the face of acutely stressful environments.

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Supplementary material cited in this article is available online.

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