Central Ghrelin Signaling Mediates the Metabolic Response of C57BL/6 Male Mice to Chronic Social Defeat Stress

Z.R. Patterson, R. Khazall, H. MacKay, H. Anisman, and A. Abizaid

Department of Neuroscience, Carleton University, Ottawa, Ontario, K1S 5B6

Chronic stressors promote metabolic disturbances, including obesity and metabolic syndrome. Ghrelin, a peptide that promotes appetite and the accumulation of adipose tissue, is also secreted in response to stressors to protect the brain and peripheral tissues from the effects of these stressors. Here we demonstrate that elevated ghrelin levels produced by chronic exposure to social stress are associated with increased caloric intake and body weight gain in male C57BL mice. In contrast, stressed mice lacking ghrelin receptors (GHSR KO mice) or C57BL mice receiving chronic intracerebroventricular delivery of the ghrelin receptor antagonist [D-Lys^3]-GHRP-6 show attenuated weight gain and feeding responses under the same social stress paradigm. Interestingly, stressed GHSR KO mice showed depleted sc and intrascapular brown fat depots, whereas stressed young wild-type mice did not. In old wild-type mice, chronic social defeat increased visceral and intrascapular brown fat depots in association with increases in obesity markers like hyperleptinemia and hyperinsulinemia along with increased hypothalamic expression of neuropeptide Y and Agouti related peptide. Importantly, the elevated expression of these peptides persisted least for 2 weeks after cessation of the stressor regimen. In contrast, old GHSR KO mice did not show these alterations after chronic social defeat. These results suggest that ghrelin plays an important role in the metabolic adaptations necessary to meet the energetic demands posed by stressors, but chronic exposure to stress-induced ghrelin elevations ultimately could lead to long lasting metabolic dysfunctions. **(*Endocrinology* 154: 1080–1091, 2013)**

In mammals, the stress response involves, among other things, the activation of the sympathetic nervous system, recruitment of the hypothalamic-pituitary-adrenal (HPA) axis, changes in neurotransmitter release in several brain regions, as well as a variety of immunological changes (1, 2). Activation of these systems facilitates the appropriate channeling of energy resources (including increased respiration, blood pressure, and the release and use of glucose stores over fat) to promote defense, escape, and ultimately survival.

In rodents these behavioral and physiological responses are associated with the adrenal steroid corticosterone (cortisol in primates). This hormone is the final product of a cascade of events that begins with the release of CRH from specialized neurons in the paraventricular nucleus of the hypothalamus (PVN) into the anterior pituitary gland. This, in turn, leads to the release of ACTH into the general circulation, which ultimately stimulates the release of glucocorticoids from the adrenal cortex. This system is under the control of a negative feedback loop, whereby elevated circulating glucocorticoids travel back to the hypothalamus (3–5), hippocampus (6, 7), medial prefrontal cortex (8, 9), and pituitary gland (10, 11) to inhibit further stimulation of the HPA axis via activation of glucocorticoid receptors (1, 2). Acutely, this response enables the behavioral and physiological adaptations necessary for the survival of an organism under stressful conditions. Repeated stressors, however, may lead to overstimulation of the HPA axis and might result in long-lasting physiological changes that ultimately lead to pathological states, includ-

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Abbreviations: AgRP, Agouti related protein; GHSR, GH secretagogue receptor; GHSR KO, stressed mice lacking ghrelin receptors; HPA, hypothalamic-pituitary-adrenal; NPY, neuropeptide Y; PVN, paraventricular nucleus of the hypothalamus; RER, respiratory exchange ratio; RT-qPCR, real-time quantitative PCR; VTA, ventral tegmental area; WT, wild type.

ing metabolic disturbances such as obesity and metabolic syndrome (1, 12, 13). Excessive glucocorticoids, for example, have been shown to cause suppression of excitatory signals (3) as well as increase inhibitory tone (14, 15) onto CRH neurons in the PVN.

In addition to corticosterone, there are other hormones that are released in response to stressors, including the gut peptide ghrelin. Ghrelin is a 28-amino acid hormonal peptide produced in the X/A-like cells of the gastric oxyntic mucosa (16). Ghrelin is best known for its ability to increase appetite and adiposity through its actions on the GH secretagogue receptor (GHSR), the only known functional ghrelin receptor (17–22). The GHSR is also expressed in the hypothalamus, with the highest expression found in the arcuate nucleus (23), a region critical for the regulation of food intake, metabolism, and energy homeostasis (19, 24). Upon binding to the GHSR, ghrelin elicits orexigenic effects by stimulating orexigenic hypothalamic peptides including neuropeptide Y (NPY) and the Agouti related protein (AgRP) (6, 8). Ghrelin promotes the use of carbohydrates as an energy substrate while sparing the use of body fat, ultimately tipping the energy homeostatic scales in favor of adiposity (19). Interestingly, plasma ghrelin levels have been shown to rise in parallel with glucocorticoids in response to both acute and chronic stressors, and ghrelin levels may remain elevated for an extended period of time after the cessation of the stressor (25–28). Because both stress and ghrelin favor the use of carbohydrates as a rapid fuel source, and given that ghrelin is secreted in response to stressors, we hypothesized that ghrelin contributes to the stress-induced metabolic switch that favors carbohydrate utilization and that ultimately leads to the accumulation of fat stores. In addition, because ghrelin influences food intake and the consumption of foods that are palatable and high in caloric content, we reasoned that ghrelin promotes the intake of these types of food in response to psychosocial stressors.

Materials and Methods

Animals

Male C57BL/J6 (The Jackson Laboratory, Bar Harbor, Maine), GHSR-KO mice and their wild-type (WT) littermates aged 8–9 weeks were used as experimental subjects. Retired breeding male CD-1 mice 13–15 weeks of age and weighing 40–50 g (Charles River Farms, St Constant, Quebec, Ontario, Canada) were used as stressors. GHSR WT and KO mice were bred at Carleton University and originated from breeding pairs obtained from Regeneron Pharmaceuticals, Inc. (Tarrytown, New York). The metabolic phenotype of this strain has been previously characterized (29). Throughout the duration of the studies, mice were housed under standard laboratory conditions and received ad libitum access to standard laboratory mouse chow and tap water as well as a daily 4-hour access to a high-fat diet containing 60% caloric content from fat (TD 06414; Harlan Teklad, Indianapolis, Indiana) to measure intake of a preferred diet. All procedures were approved by the Carleton University Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care.

Chronic social defeat paradigm

Mice were matched for body weight and caloric intake after a 21-d baseline period wherein they had ad libitum access to tap water and regular mouse chow as well as 4-hour access to a high-fat diet from 10:00 AM to 2:00 PM. After the baseline period, mice were assigned to either the stress or nonstress groups. Animals in the stress groups were housed in a separate room and subjected to the resident intruder paradigm to induce chronic social defeat stress (30, 31). In this paradigm, mice were housed with the same CD-1 mouse resident for the entire 21-day stress period. CD-1 mice were screened for aggression before the experiment and established dominance within 2–3 days of the stress period. A central divider made from transparent acrylic and wire mesh was used to prevent any physical contact between the two animals, but permitted the exchange of sensory information. Each day, at 2:00 PM the divider was removed to allow interaction. The divider was replaced once the CD-1 mouse subdued the experimental mouse, or after 15 minutes had passed. After the stress period, all animals were allowed to recover for 3 weeks (returning to baseline conditions) before being humanely killed unless otherwise stated. Control animals were housed in cages without the dividers given pilot data showing that food intake and body weight of nonstressed WT and GHSR mice housed in cages with dividers does not differ from that of mice housed without dividers (see Supplemental Figure 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Chronic intracerebroventricular delivery of ghrelin antagonist

After a baseline period of 2 weeks, mice were assigned to groups that were matched for average body weight and caloric intake and implanted with a 31-gauge stainless steel L-shaped cannula (Alzet Brain Infusion Kit no. 0004760) coupled to an osmotic mini-pump (Alzet Mini-Osmotic Pump model 2ML4; flow rate: 0.25 μl/h for 28 d) via a polyethylene catheter under isofluorane/oxygen (4%) anesthesia. The cannula was aimed at the left lateral ventricle [coordinates: anterioposterior, −0.10 mm, mediolateral, −0.80 mm, and dorsoventral, 2.20 mm; (32)]. Minipumps were filled with 240 μl of either sterile saline (0.9% NaCl) or a ghrelin receptor antagonist ([D-Lys3]-GHRP-6) solution (Peptides International; 20 nmol/d/mouse). The cannula was held in place secure to the skull using contact and dental cement. After 1 week of recovery after surgery, mice were started on the 21-day experimental stress paradigm. Mice were then humanely killed by intracardial perfusion with ice-cold saline, followed by 4% paraformaldehyde. Brains were collected and sliced to confirm the accuracy of the cannula placements. Mice with missed placements were not included in the analyses.

Indirect calorimetry

Measurements of oxygen consumption (VO_2), carbon dioxide production (VCO_2), and respiratory exchange ratio (RER) were performed using phenomaster/labmaster metabolic cages (TSE instruments, Chesterfield, Missouri). Seven days after the last stressor, mice were housed individually in the chambers for 48 hours, with regular chow and water available ad libitum. The high-fat diet was also available during the same time period because it was in their home cages. The software recorded oxygen, carbon dioxide, food intake, water intake, and locomotor activity every 30 minutes for 48 hours. Only the recordings from the latter 24 hours were used for the analyses.

Tissue processing

Animals were killed by rapid decapitation unless otherwise stated, and plasma and tissue samples were collected and frozen at −80°C. To measure glucose levels, glucose strips attached to a Contour glucose meter (Bayer Corp., Pittsburgh, Pennsylvania) were dipped in trunk blood collected before being centrifuged. Brains were rapidly dissected and tissue micropunches of the mediobasal hypothalamus [1.5–2.0 mm behind bregma (32)] were collected using hollow 16-gauge needles with a beveled tip as described by Palkovits (33). The collection of these punches took no longer than 2 minutes after the decapitation of the animal. Finally, carcasses were frozen at −80°C until they were dissected to obtain different fat pad weights. In addition, samples of the perigonadal fat were collected and stored in Trizol at −80°C for histological or real time-quantitative PCR (RT-qPCR) analysis.

Hormone analyses

After animals were humanely killed, trunk blood was collected in EDTA-coated tubes placed on ice and centrifuged at 3000 × *g* for 15 minutes to separate plasma from red blood cells. Blood plasma was aliquoted separately for each assay to avoid multiple freeze/thaw cycles, and stored at −80°C until processed. To protect the acylated ghrelin molecule, a 50-μl aliquot of blood plasma was treated with 2.7 μl of 1.0 N HCl and 10 μl of 100 mM 4-(hydroxymercuri) benzoic acid before storage. Plasma corticosterone levels were measured in duplicates using a commercially available RIA kit (ICN Biomedicals, Inc, Aliso Viejo, California). Plasma insulin, leptin, resistin, and IL-6 were measured using a mouse adipokine milliplex kit (Mouse Serum Adipokine Immunoassay; Millipore Corp., Bedford, Massachusetts). Plasma acylated ghrelin and adiponectin were measured using an ELISA kit (Millipore). All samples had a coefficient of variation < 10 and 15%, respectively).

Histology

To analyze adipocyte morphology, paraffin-embedded perigonadal fat samples were sliced at 8 μm and stained with hematoxylin and eosin. Adipocyte diameter was measured using ImagePro software (Media Cybernetics, Bethesda, Maryland). A total of 200 randomly selected adipocytes were used per animal.

RT-qPCR

Total RNA from brain and white adipose tissue was isolated with TriZol and precipated with 13 μl of linear acrylamide. RNA quality and concentrations were determined by absorbance at 280 nm and 260 nm with a Thermo Scientific Nanodrop 100 spectrophotometer (Thermo Scientific, Rockford, Illinois). To synthesize cDNA, 1 μl oligo(dT) primer (Invitrogen, Carlsbad, California) was added to 9 μl of mRNA and heated to 70°C for 5 minutes. To each sample, a master mix composed of 4 μl of 5× first-strand buffer (Invitrogen), 2 μl of 0.1 M dithiothreitol (Invitrogen), 1 μl of RNase inhibitor (Promega Corp, Madison, Wisconsin), 1 μl of 10 mM deoxynucleotide triphosphate (Invitrogen), 1 μL of diethylpyrocarbonate water, and 1 μl of SS2 reverse transcriptase (Invitrogen) were added. Samples were then run on a PTC-200 Thermal Cycler (MJ Research, Watertown, Massachusetts) at 42°C for 1.5 hours followed by 90°C for 10 minutes Samples were stored at −20°C. RT-qPCR was conducted on all cDNA samples to determine fold changes using the $2^{-\Delta\Delta Ct}$ method using primers detecting the glyceraldehyde 3-phosphate dehydrogenase gene as a control transcript (34). Briefly, 5 μl of each cDNA sample were added to separate wells in a PCR plate. Two microliters of working primer solution, 3 μl of Milli-Q water, and 10 μl of iQ SYBR Green Super Mix with fluorescein (Bio-Rad Laboratories, Inc, Hercules, California) were added to each well. Samples were run in duplicate, with nontemplate controls. The plate was run on a MyIQ Single Color Real-Time PCR Detection System (Bio-Rad) for 30 sec at 95°C, followed by 45 cycles of the following settings: 10 sec at 95°C for denaturing, 30 sec at 55°C for annealing, and 20 sec at 72°C for extension. The plate was then run for 1 minute at 95°C and 1 minute at 55°C. The nucleotide sequences of the various primers used are provided as Supplemental Table 1. All primers were tested for amplification efficiency using the standard curve method, yielding efficiencies over 90%.

Results

Chronic social defeat increases plasma ghrelin concentrations and causes long lasting changes in caloric intake and body weight

Social stressors affect food intake, body weight, body composition, and lipid metabolism in rodents and nonhuman primates (30, 31, 35–40). In support of these studies, Figure 1 shows ghrelin concentrations, caloric intake, and weight gain of stressed and nonstressed mice after 3 weeks of chronic social defeat, and after a 3-week recovery period. As seen in this figure, ghrelin concentrations were elevated in C57BL/J6 mice at the end of a 3-week chronic social defeat stress paradigm ($t_{(13)} = -2.89$, $P < .05$), and in parallel with morning corticosterone concentrations (CORT; $t_{(13)} = -3.89$, $P < .05$; see Figure 1). During baseline, high-fat diet consumption increased gradually, and in parallel with a gradual decrease in the intake of regular chow (see Figure 1), indicating that the mice developed a preference for this diet. During the stress period, however, socially defeated mice increased their total caloric intake ($F_{(1, 23)} = 6.24$, $P < .05$), their regular chow intake ($F_{(1, 23)} = 9.41$, $P < .05$), and weight gain ($F_{(1, 23)} = 17.61$, $P < .05$) despite a decrease in the consumption of the calorically dense high-fat diet ($F_{(1, 23)} = 35.97$, $P <$

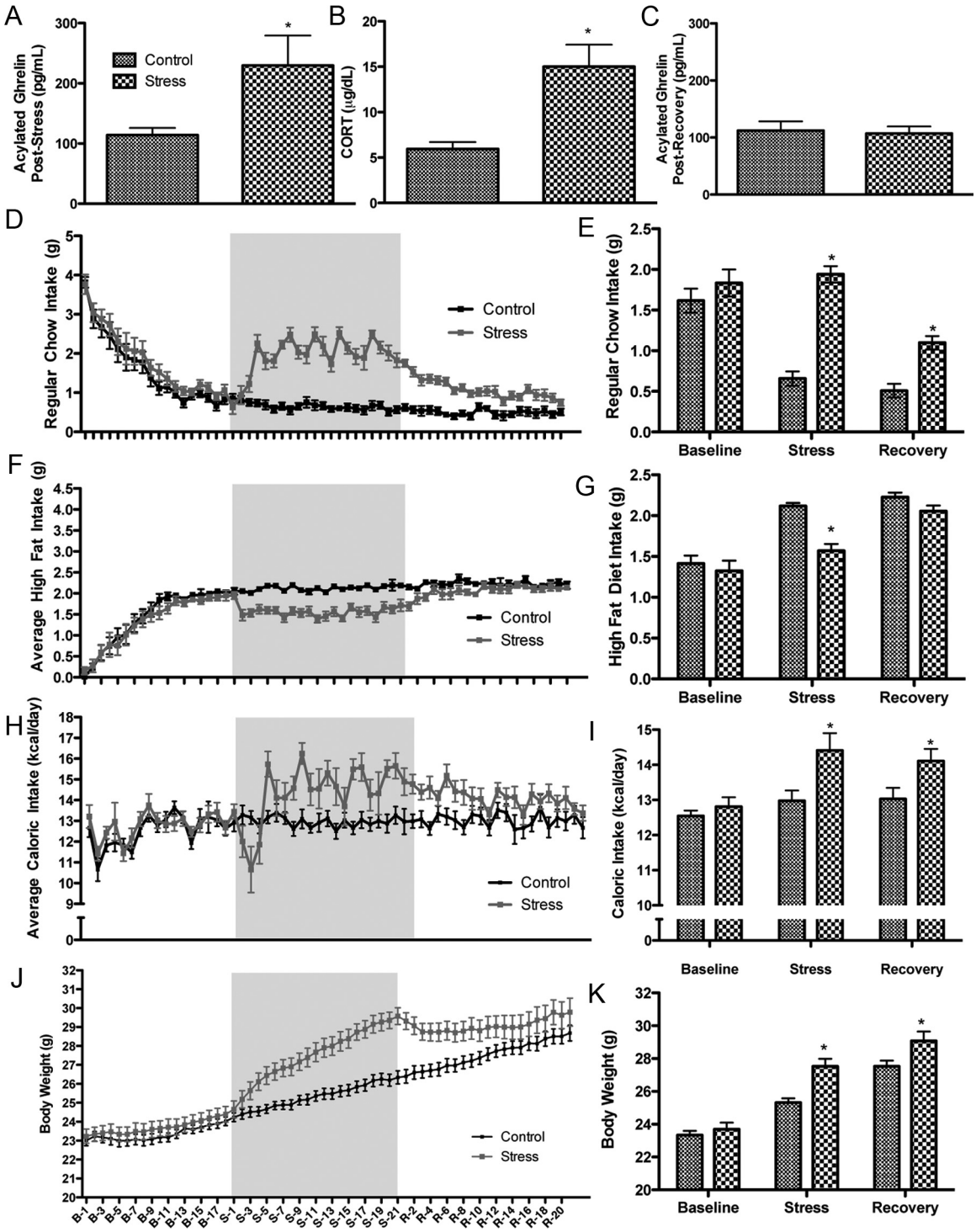

Figure 1. Acylated Ghrelin Levels 24 hour after Stress (A), Plasma Corticosterone Levels 24 hour after Stress (B), Plasma Acylated Ghrelin 21 days after Their Last Social Defeat Episode (C), Daily Regular Chow Consumption (D), Average Regular Chow Consumption (E), Daily High-Fat Diet Intake (F), Average High-Fat Diet Intake (G), Daily Caloric Intake (H), Average Caloric Intake (I), Daily Body Weights (J), and Average Body Weights (K) for Stressed and Nonstressed Controls during the Baseline Period, Stress Period, and Recovery Period. All values are expressed as mean ± SEM. Baseline period is denoted as B1–B21; stress period is denoted as S1–S21 and is illustrated with shaded box, and recovery period is denoted as R1–R21. * $P < .05$ relative to nonstressed controls

.05). More importantly, the food intake and body weight of stressed mice did not return to control levels for another week after the stress ($P < .05$). At the end of the recovery, plasma concentrations of active ghrelin in socially defeated mice did not differ from those of nonstressed mice ($F_{(1, 23)} = 0.05$, $P > .05$; See Figure 1).

The effects of chronic social defeat on body weight and food intake are GHSR dependent and central in origin

To determine the role of ghrelin in mediating the effects of stress on body weight gain and caloric intake, we assessed the impact of chronic social defeat in GHSR KO mice as well as their WT littermates. As in the previous study, chronic social defeat caused a significant increase of acylated ghrelin concentrations, coupled with an increase in body weight gain and a tendency to increased caloric intake in WT mice; these effects were attenuated in GHSR KO mice (see Figure 2). However, chronic social defeat increased plasma corticosterone levels similarly in both GHSR KO and WT mice, indicating that the feeding and body weight increases seen in WT mice were due to increases in ghrelin and not corticosterone (see Figure 2).

To determine whether these effects are mediated by the action of ghrelin at central sites, we assessed the effects of the ghrelin receptor antagonist [D-LYS^3]-GHRP-6 vs vehicle administered into the left lateral ventricle of mice equipped with osmotic minipumps connected to an intracranial cannula delivery system. Half of the mice in each group were exposed to the chronic social defeat stress paradigm, while the other half served as nonstressed controls. As expected, stressed animals infused with the vehicle increased both their total caloric intake and body weight gain ($F_{(3, 21)} = 4.45$, $P < .05$, and $F_{(3, 21)} = 4.86$, $P < .05$, respectively; see Figure 3) relative to their nonstressed controls. Although stressed mice treated with the ghrelin receptor antagonist also increased their food intake and body weight, the extent of the increase was attenuated and was significantly lower than the increases observed in stressed vehicle-treated mice (see Figure 3).

Chronic social defeat favors the use of carbohydrates in WT but not in GHSR KO mice

Chronically elevated levels of ghrelin promote the use of carbohydrates as a fuel source while conserving fat stores, metabolic changes that lead to increased adiposity (19). Given that chronic social defeat stress causes an increase of plasma acylated ghrelin concentrations, we hypothesized that this treatment would cause a metabolic change promoting carbohydrate use while sparing fat stores, and that this would not be evident in GHSR KO mice. To this end we housed GHSR KO and WT mice in metabolic chambers to obtain measures of metabolic parameters using indirect calorimetry. During the baseline period, all mice showed equivalent metabolic phenotypes on all measures taken (data not shown). After the chronic social defeat paradigm, mice were allowed to recover for 2 wk before being placed back into the metabolic cages. As shown in Figure 3, the RER, an index of energy substrate utilization, was higher in WT stressed mice compared with WT controls, an effect that did not occur in GHSR KO mice ($F_{(1, 31)} = 3.95$, $P < .05$). Given these data, we suspected that stressed GHSR KO mice would show depletion of fat stores. An analysis of fat pad weights after death indeed revealed that GHSR KO mice had the lowest total

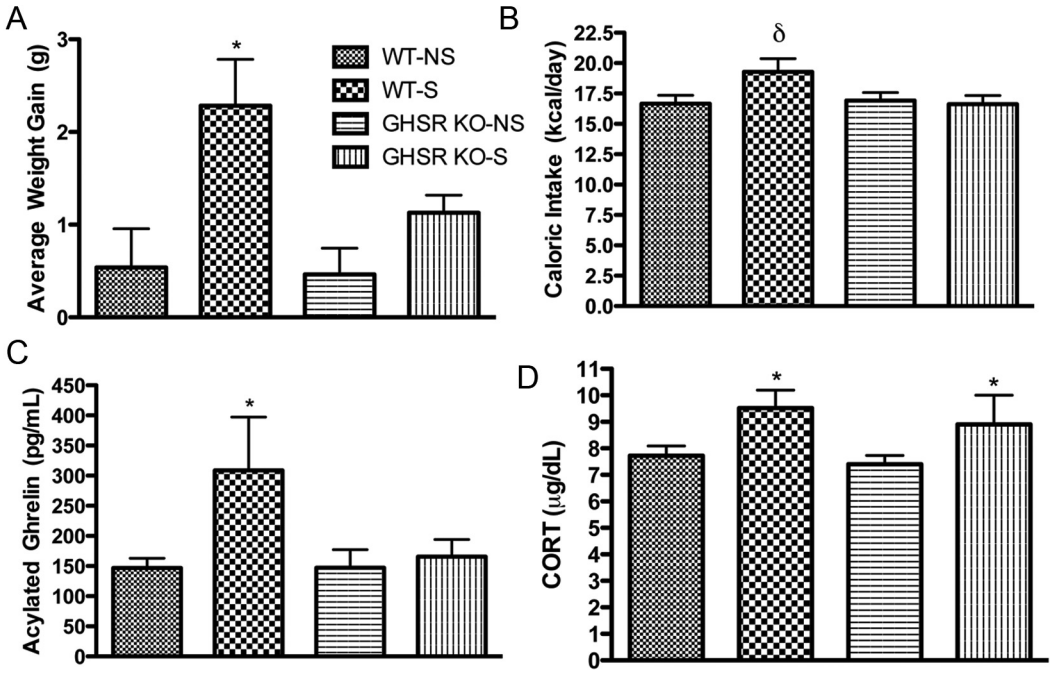

Figure 2. Weight Gain (A) and Caloric Intake (B) during the Chronic Social Defeat Stress Paradigm. Acylated ghrelin levels (C) and corticosterone (CORT) (D) from trunk blood collected at death. All values are expressed as mean ± SEM. * $P < .05$; δ $P < .10$ relative to nonstressed controls. NS, nonstressed; S, stressed.

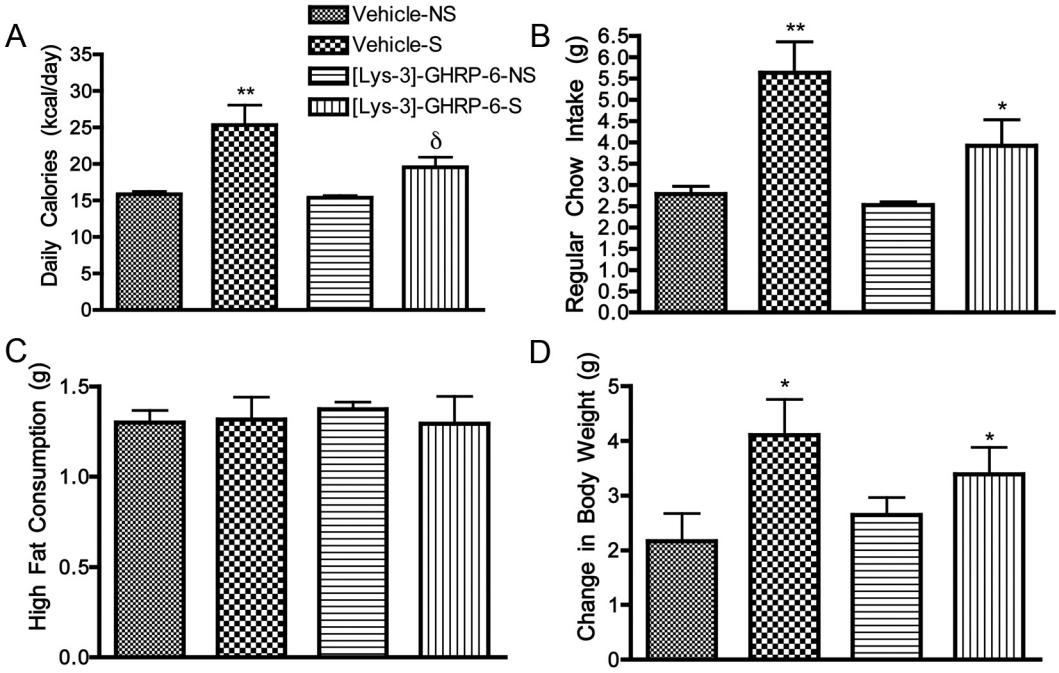

Figure 3. Metabolic Profile of Animals Receiving Central Infusions of Either Vehicle (Saline) or a Ghrelin Receptor Antagonist ([D-Lys3]-GHRP-6) in Response to Chronic Social Defeat. Average daily caloric consumption (A), chow consumption (B), high-fat diet consumption (C), and average change in body weight during the stress period (D). All values are reported as mean ± SEM. ** $P < .01$; * $P < .05$; δ $P < .10$ vs nonstressed controls. NS, nonstressed; S, stressed.

fat pad weights, although only the differences in intrascapular brown fat pad and sc fat pad weights attained statistical significance ($F_{(3, 38)}$ values = 7.37 and 4.18, $P < 0.05$, respectively; see Figure 4).

The divergent metabolic consequences of chronic social defeat stress are exacerbated in aged WT and GHSR KO mice

The expression of GHSR in adipose tissues is very low in young mice, but increases as the mice age, and this increase has been associated with increased adiposity and altered metabolism (41). Because of this, we suspected that chronic social defeat would have a greater impact on metabolism in mice that were older than those we used in the previous study. We therefore exposed 6- to 7-month-old GHSR KO and WT mice to the chronic social defeat stress and allowed them to recover for an additional 3 weeks before being humanely killed.

Results from this study showed that chronic social defeat caused an increase in visceral (perigonadal/inguinal) fat pad and in intrascapular brown fat pad weights in WT, but not in GHSR KO mice, relative to their nonstressed

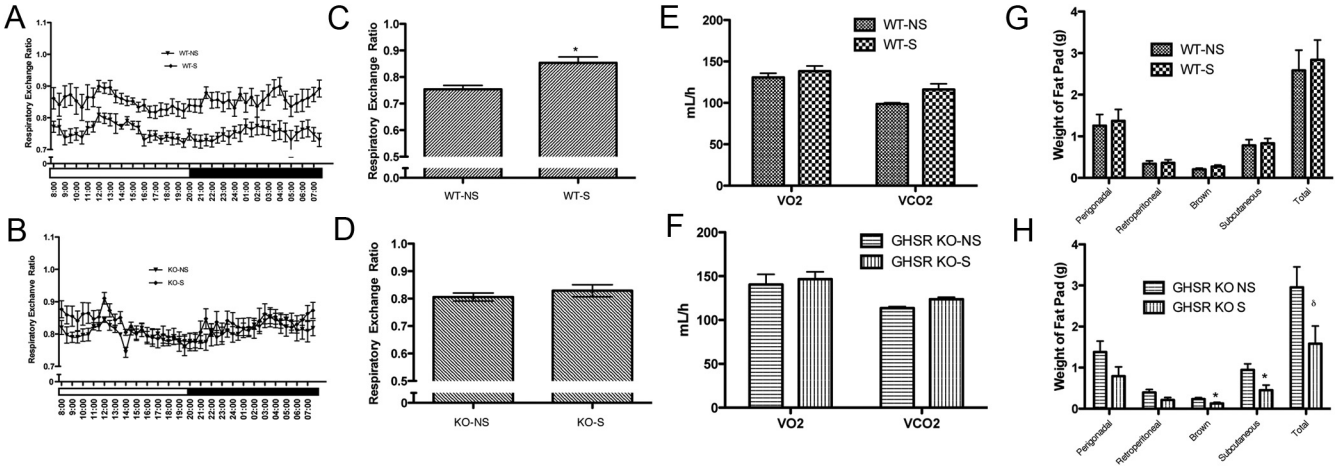

Figure 4. RER Measured via Indirect Calorimetry during the Recovery Period vs. Time of Day for WTs (A) and GHSR KOs (B), Average Daily RERs for WTs (C) and GHSR KOs (D), Average Oxygen Consumption and Carbon Dioxide Production for WTs (E) and GHSR KOs (F) and Fat Pad Weights for WTs (G) and GHSR KOs (H). All values are reported as mean ± SEM. * $P < .05$; δ $P < .10$ vs nonstressed controls. NS, nonstressed; S, stressed.

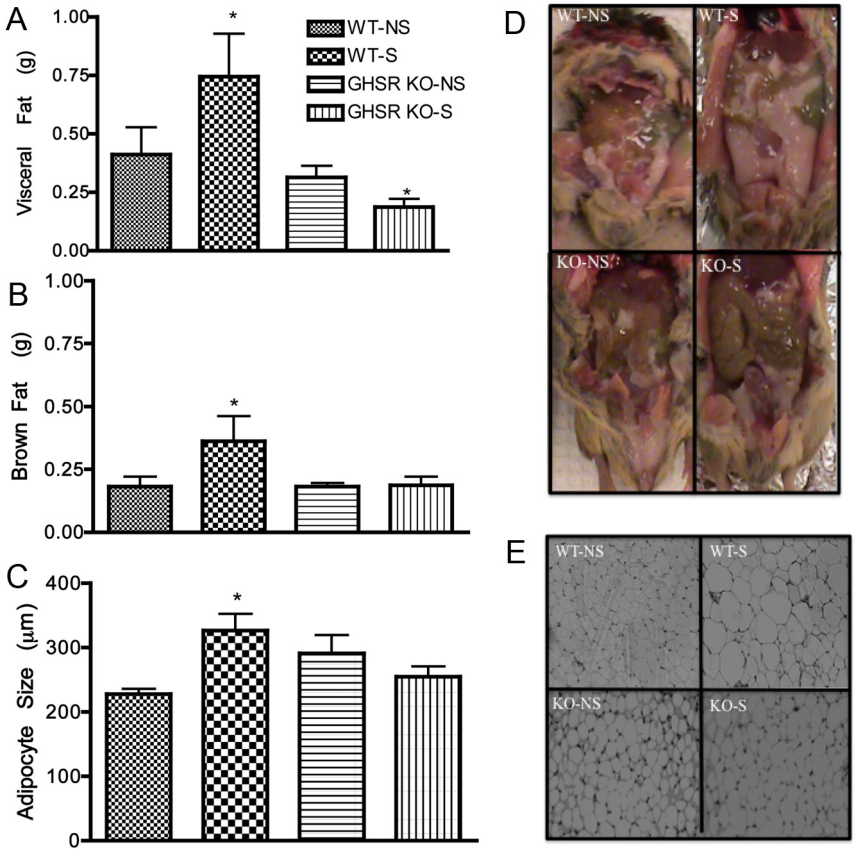

Figure 5. Average Weight Visceral Fat (A), Average Weight of Intrascapular Brown Fat (B), and the Average Diameter of Visceral Adipocytes (C). Illustration of visceral adipose tissue (D) and average diameter of visceral adipocytes (E).* $P < .05$ vs nonstressed controls. NS, nonstressed; S, stressed.

controls ($F_{(3, 36)}$ values = 4.69, 4.57; P values < .05, respectively; see Figure 5). Histological analyses from visceral fat samples showed that WT stressed mice had significantly larger adipocytes than WT nonstressed mice, with no differences existing between stressed and nonstressed GHSR KO mice ($F_{(3, 33)} = 8.60$; $P < .05$; see Figure 5). In concert with these measures of adiposity, WT stressed mice also showed markers of obesity such as hyperinsulinemia ($F_{(3, 30)} = 8.57$; $P < .05$), elevated basal corticosterone levels ($F_{(3, 35)} = 3.44$; $P < .05$) and an increase of IL-6 ($F_{(3, 33)} = 5.80$; $P < .05$; see Figure 6) relative to their nonstressed controls. Furthermore, WT mice tended to have higher levels of blood glucose and leptin relative to GHSR KOs ($F_{(3, 31)} = 3.33$; $P < .10$ and $F_{(3, 25)} = 2.98$; $P < .10$ respectively; see Figure 6).

Gene expression analyses of white adipose tissues using RT-qPCR showed that visceral white adipose tissue obtained from stressed GHSR KO mice had decreased expression of fatty acid synthase, an enzyme important for the anabolic processes associated with storing fat as well as an enzyme that is decreased after fasting and ghrelin treatment ($F_{(3, 28)} = 3.38$; $P < .05$; Supplemental Figure 1). Other transcripts associated with lipid handling such as lipoprotein lipase, acetyl CoA carboxylase, and the NPY Y2 (Y2-R) receptor were not different between the strains of mice and were unaltered after chronic social defeat (Supplemental Figure 1). The metabolic profile of WT stressed mice was positively correlated with the expression of hypothalamic peptides that are elevated during negative energy balance. For instance, WT stressed mice had increased hypothalamic NPY ($F_{(3, 28)} = 7.21$; $P < .05$); and AgRP gene expression ($F_{(3, 28)} = 4.13$, $P < .05$; see Figure 6).

Discussion

Physiological responses to stress include a number of endocrine changes that affect both the periphery and the brain. In the short term these changes allow for an organism to generate sufficient energy to meet the challenges presented by the stressors. Over prolonged periods of time, however, these protective mechanisms may themselves become pathological (1). Here we present data suggesting that ghrelin, a metabolic hormone that is released after stress to protect the periphery and the brain, can ultimately lead to metabolic changes that promote obesity in the face of chronic exposure to stress. Furthermore, these effects may be mediated, in part, by the action of ghrelin in the brain.

The protective effects of ghrelin have been highlighted in a number of studies using both acute and chronic stress paradigms. Indeed, ghrelin is secreted in response to acute stressors (26, 42), and exogenous ghrelin treatment evokes the release of ACTH and prolactin from the pituitary (43, 44). Chronic stressors also increase ghrelin concentrations that persist for some time beyond the duration of the stress protocol (27, 45). Peripherally, ghrelin seems to protect the stomach against ulceration that occurs after chronic stress (27). However, ghrelin serves a number of functions other than gastro-protection after stress. For instance, ghrelin attenuates the display of anxiety and depressive-like behavior in rodents (45–47). In contrast, GHSR KO mice display more of these behaviors after 10 d of daily social defeat (45). In all, these data suggest that ghrelin signaling during stress is important to counter the ef-

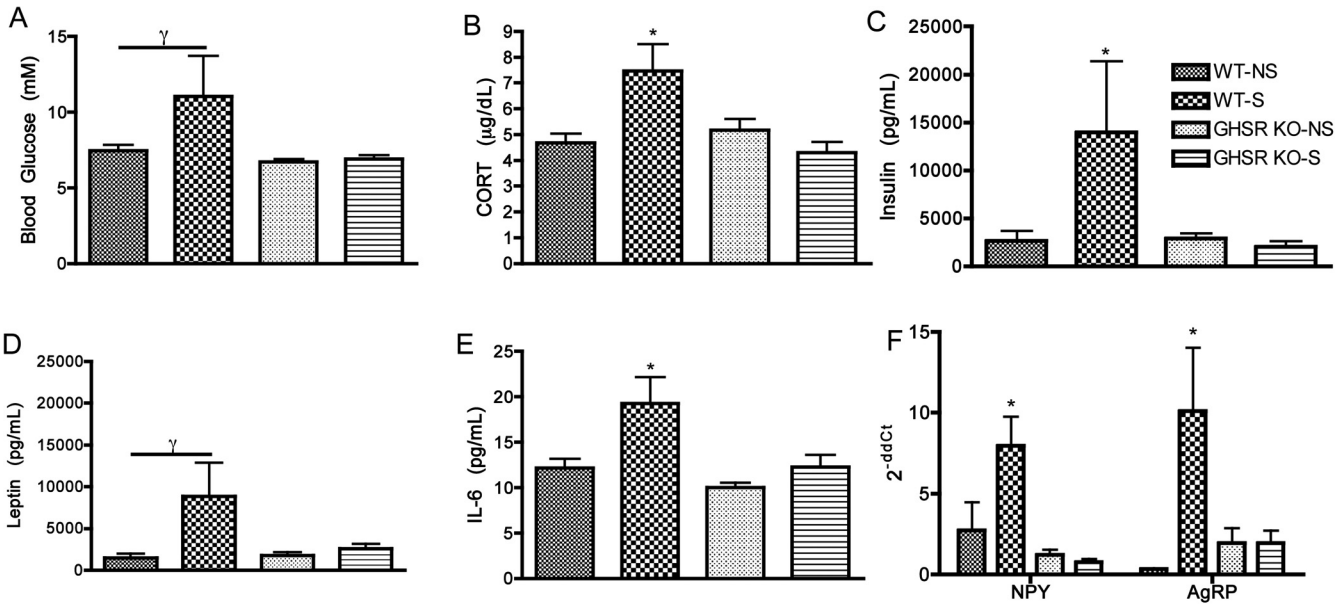

Figure 6. Hormonal Profile of Trunk Blood Collected at Time of Death Mean concentration of plasma blood glucose (A), corticosterone (CORT) (B), insulin (C), leptin (D), and IL-6 (E). All values are expressed as mean ± SEM. mRNA expression of NPY and AgRP in the mediobasal hypothalamus (F). All values are expressed as mean ± SEM, relative to GAPDH expression. * $P < .05$ relative to nonstressed controls; γ $P < .10$ vs GHSR KOs. NS, nonstressed; S, stressed.

fects of chronic stressors, and the lack of ghrelin signaling may lead to psychopathological conditions such as depression (45).

The protective effects of ghrelin could come from promoting energy replenishment, where ghrelin promotes an increase of appetite to offset the energetic costs of dealing with a stressful event. This effect has been reported previously (45, 48) and is confirmed in our current studies in which exposure to chronic social defeat in mice produced increased plasma acylated ghrelin concentrations, and this occurred in tandem with increased caloric intake and body weight. Unexpectedly, the stress-induced increase in caloric intake did not come from an increase in the consumption of the calorically dense diet that the animals clearly preferred before the stress paradigm began. During the baseline, mice in our studies clearly increased their intake of the high-fat diet when it became available, while decreasing their intake of regular chow. When stressed, however, mice clearly decreased their intake of the high-fat diet, but increased their intake of the regular chow, an effect that was slowly reversed during the recovery period. Although we expected that stressed mice would eat more of this preferred diet in anticipation of the stressor, they ate less. In contrast, others have shown that mice increase the intake of high-fat pellets, and show stronger conditioned place preferences to these pellets after chronic social defeat stress, whereas GHSR KO mice do not show these preferences (36). One possibility for the discrepancy between our results and those of others (ie, Ref. 36) may have to do with the timing and the context in which the preferred diet is presented. Thus, although mice may engage in increased reward-based eating and develop conditioned place preferences to palatable foods when tested after being stressed (36), this may not be the case if tested while they are being stressed, as is the case in our studies, where they clearly show a decrease in their preference for the high-fat diet. Moreover, it is possible that in our studies, stressed mice associated the presence of the diet with the oncoming stressor, ultimately reducing the intake of the high-fat diet. Hours after the stress bout has occurred, but when the high fat is no longer available, stress-induced ghrelin secretion produced an increase in chow intake that was significant enough to increase total caloric intake.

A second protective mechanism would be one in which ghrelin favors the utilization of carbohydrates to meet the rapid energetic challenges posed by a chronic stressor. In the present studies we show that WT stressed mice favor the use of carbohydrates as a fuel source, while sparing fat utilization as reflected in a higher RER compared with nonstressed mice. This difference, however, was absent in GHSR KO mice. Interestingly, stressed GHSR KO mice tended to have the lowest amounts of fat pads overall, and this was particularly evident in the brown and sc adipose depots. Thus, it appears that GHSR KO stressed mice use more of these depots as a fuel source relative to WT stressed mice, or that they are less capable of storing fat while stressed. The change in body composition seen in GHSR KO stressed animals is likely a product of their energy substrate utilization and not due to differences in food intake. For instance, young WT stressed animals

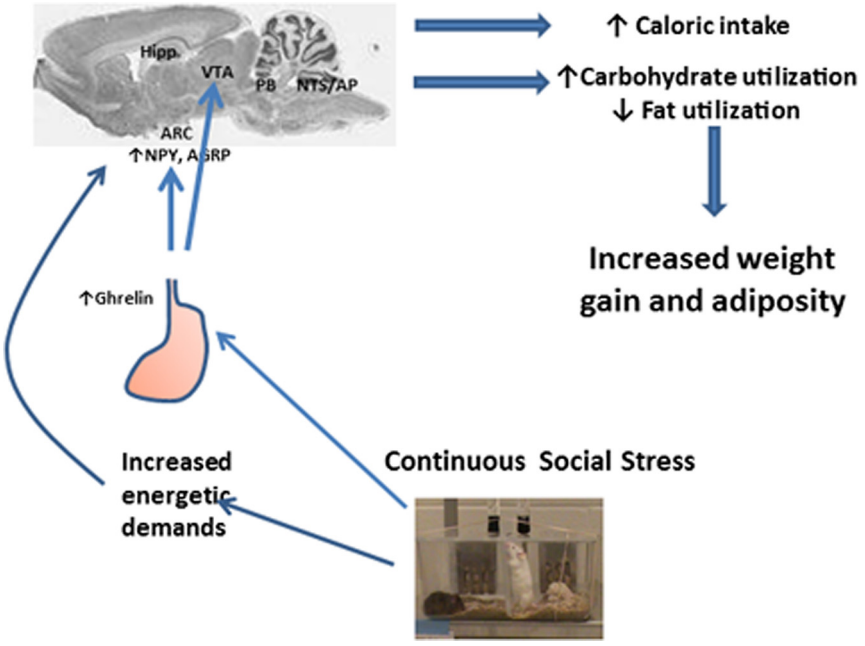

Figure 7. Proposed Explanation of How Ghrelin Mediates the Metabolic and Behavioral Adaptations Necessary to Deal with Stress. Chronic stress produces an energetic demand that is met by increased caloric intake and by switching metabolism to favor the utilization of carbohydrates over fat as a source of fuel. This effect is mediated by ghrelin, and lack of GHSR prevents this metabolic switch from occurring. In the long run chronically increased levels of ghrelin may lead to obesity and metabolic disorders.

showed an increase in caloric intake relative to their nonstressed controls, but no differences in body composition. In contrast, GHSR KO stressed animals do not show changes in caloric intake compared with their nonstressed controls, but do show a reduction in fat deposition, supporting the notion that they continue to use fat depots as fuel. If differences in food intake were to dictate the overall accumulation of adipose tissue, one would expect stressed WT mice to show increased adiposity whereas GHSR KO mice would not show a drop in adipose tissue because they continued to eat the same as in the baseline.

Stress-induced increases in caloric intake, along with decreased use of fat as fuel, would be expected to result in increased adiposity over time. Young WT mice in our studies, however, did not show long-term increases in fat accumulation after the stress paradigm, suggesting that, despite stressor-induced increases of food intake, body weight, and RER, they ultimately recovered and did not accumulate more body fat than nonstressed animals. In contrast, older mice WT mice showed clear signs of metabolic imbalance following the same chronic social defeat paradigm. Among these, these mice showed increased accumulation of visceral white adipose tissue, as well as increased intrascapular brown fat mass. In contrast, stressed GHSR KO mice had the lowest accumulation of adiposity in these same regions. Older stressed WT mice showed adipocytes that were significantly larger in size than those of nonstressed WT mice, a difference not seen in GHSR KO mice. Although these are crude measures of adiposity, and a more accurate measure of body composition is required, they do correlate well with other measures of adiposity, such as higher plasma concentrations of leptin, insulin, glucose, and IL-6, a difference that was absent in GHSR KO animals. Finally, the hypothalamus of WT stressed mice showed increased NPY and AgRP mRNA expression, whereas hypothalamic gene expression among GHSR KO mice remained equivalent to that of their nonstressed controls. It is important to note that these plasma analyses were obtained from samples collected 2 weeks after the last stressor. Therefore, not only are older WT mice less able to maintain metabolic homeostasis during the chronic stress paradigm, but these alterations are long lasting. More importantly, old GHSR KO mice did not show these alterations, suggesting that ghrelin is responsible for these alterations.

Ghrelin's effect on food intake and metabolism are mediated by the action of ghrelin on the GHSR both in the brain and in white adipose tissue (19, 41). Effects of ghrelin directly on adipose tissues, however, are more pronounced as animals (including humans) age. It has been suggested that increases in adiposity in older animals is a function of increased adipose tissue GHSR expression in older animals (41). Our data do suggest that the effects of chronic social defeat on energy balance are mediated, in part, by central ghrelin signaling. Indeed, GHSR KO mice, and mice treated chronically with intracerebroventricular delivery of [D-Lys-3]-GHRP6 during the stress period, showed attenuated feeding and weight gain responses, while maintaining elevated concentrations of corticosterone. There are several central sites at which ghrelin could act to increase food intake and body weight during chronic social defeat, because ghrelin receptors are located in a number of brain sites known to be part of both the regulation of energy balance and stress including the PVN, hippocampus, ventral tegmental area (VTA), the edinger-westphal nucleus, the nucleus of the solitary tract (NTS) in the brain stem, and the amygdala (46, 48, 50–53); however, the relative contributions of each to stress-induced feeding remains to be determined. For instance, ghrelin

could be acting directly onto the PVN to alter substrate utilization and favor carbohydrate metabolism (52). Ghrelin may act on the hippocampus to protect against damage from chronic glucocorticoid exposure after chronic social defeat while promoting the effectiveness of cues that predict the availability of foods to elicit feeding (48, 54). In the VTA, ghrelin may increase the motivation to eat palatable foods after chronic social defeat particularly through the stimulation of VTA dopamine neurons (36, 53, 55–57). In the edinger-westphal nucleus, ghrelin could increase urocortin release, which, in turn, could modulate feeding and lipid metabolism after chronic stress (42, 58). Finally, stress-induced ghrelin secretion may alter sympathetic responses and glucose-regulatory processes by actions on GHSRs located in the nucleus of the solitary tract (59).

There are some limitations in our study that must be considered. For instance, the ghrelin receptor antagonist used in this study is not entirely selective to GHSR (60, 61). It has been shown that [D-Lys^3]-GHRP6 is also capable of acting as an agonist to the 5-HT$_{2b}$ receptor to influence the contraction of fundic strips (60). Furthermore, [D-Lys^3]-GHRP6 is also capable of regulating chemokine signaling through interactions with CXCR4 in immune cells (61). Whether similar effects occur centrally has not yet been determined, and given that antagonist-treated mice show a profile similar to that of GHSR KO mice when exposed to the chronic social defeat, we tend to attribute these effects to the influence of [D-Lys^3]-GHRP6 on the GHSR and not to serotonin or chemokine receptors. However, future studies need to be conducted to determine this as better GHSR antagonists become more readily available.

It is also important to note that the endocrine responses seen after the chronic stress period were not as marked as expected, particularly in mice killed 24 hours after their last stressor. For instance, and regardless of their genotype, stressed mice showed significantly higher corticosterone levels, but these were only about 10% higher than controls. In addition, GHSR KO mice did not have increased ghrelin concentrations 24 hours after their last stressor, whereas WT mice did. This is in contrast with studies using a chronic social defeat paradigm similar (but not the same) to the one used here, or using chronic unpredictable stressors, wherein increased plasma ghrelin concentrations were seen in GHSR KO mice (36, 49). Given that in our studies stressed mice are exposed to the same aggressive male for 21 d, the lower than expected corticosterone levels seen in stressed mice, and/or lower ghrelin concentration observed in stressed GHSR KO mice, may reflect habituation to the familiar aggressive males (62). This also would explain differences in corticosterone seen after the social defeat paradigm that we used here and those seen by our group when using a chronic unpredictable stressors paradigm (49). In spite of this, basal corticosterone levels from mice in these studies were elevated and maintained for at least 2 weeks after the end of the stress, suggesting that our stress protocol was not only sufficient to elicit the activation of the HPA axis, but that this system continued to be excited for several days after the stress protocol was terminated.

Overall these data suggest that stress produces an increase of ghrelin that increases food intake and promotes the utilization of carbohydrates over fat, in order to meet the energetic challenge posed by the stressor. This allostatic change is helpful in the short term, but in the long term it has an impact on metabolism, particularly in older mice whose ability to maintain energy balance is compromised (see Figure 7). Stressed mice with mutations to the GHSR gene do not show these allostatic changes. Instead, GHSR KO mice meet the energetic demands of the chronic stressor by increasing the use of fat as a fuel source. This physiological change, however, may be insufficient to protect these mice against the effects of chronic stress and may make them more vulnerable to stress-induced depression (45).

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Address all correspondence and requests for reprints to: Alfonso Abizaid, Department of Neuroscience, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, K1S 5B6. E-mail: alfonso_abizaid@carleton.ca.

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