Des-acyl ghrelin and Ghrelin O-Acyltransferase regulate Hypothalamic-Pituitary-Adrenal axis activation and anxiety in response to acute stress

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Ghrelin exists in two forms in circulation, acyl ghrelin and des-acyl ghrelin, both of which have distinct and fundamental roles in a variety of physiological functions. Despite this fact, a large proportion of papers simply measure and refer to plasma "ghrelin" without specifying the acylation status. It is therefore critical to assess and state the acylation status of plasma ghrelin in all studies. In this study we tested the effect of des-acyl ghrelin administration on the HPA axis and on anxiety-like behaviour of mice lacking endogenous ghrelin, and in GOAT KO mice that have no endogenous acyl ghrelin and high endogenous des-acyl ghrelin. Our results show des-acyl ghrelin produces an anxiogenic effect under non-stressed conditions but this switches to an anxiolytic effect under stressed. Des-acyl ghrelin influences plasma corticosterone under both non-stressed and stressed conditions although c-fos activation in the PVN is not different. By contrast, GOAT KO are anxious under both non-stressed and stressed conditions although this is not due to corticosterone release from the adrenals but rather from impaired feedback actions in the PVN, as assessed by c-fos activation. These results reveal des-acyl ghrelin treatment and GOAT deletion have differential effects on the HPA axis and anxiety-like behaviour, suggesting that anxiety-like behaviour in GOAT KO mice is not due to high plasma des-acyl ghrelin.

Since its discovery in 1999, the peptide hormone ghrelin has received significant research attention, particularly because of its strong effect to increase food intake, and regulate glucose homeostasis and body weight (1). Ghrelin is a 28 amino acid peptide hormone secreted primarily from the stomach and digestive tract (2) in response to sympathetic activation of β-adrenergic receptors (3). A large body of evidence has emerged to show that acyl ghrelin functions as a key hormone conveying negative energy balance back to the brain, with diminished functional efficacy in states of positive energy balance (4–7). However, acyl ghrelin also performs numerous nonmetabolic functions. These include regulating cognitive function, reward, neuroprotection, pain, vascular function, anxiety and stress (8–15).

In particular, acyl ghrelin's role to regulate the hypothalamic pituitary adrenal (HPA) stress axis and control an appropriate stress response has garnered significant attention. For example, all types of stress, whether they are physical or psychological, increase plasma acyl ghrelin,

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Abbreviations:

ghrelin messenger RNA (mRNA) and/or ghrelin cell numbers in the stomach (16–21). Even the anticipation of a stressor increases plasma acyl ghrelin in humans compared to the control condition (22). Moreover, intraperitoneal injection of acyl ghrelin produces a significant dose-dependent increase in serum corticosterone levels one hour after injection (16). In response to stress, activation of the HPA axis enables the body to respond appropriately to deal with the stressor. Inappropriate chronic activation of HPA axis can lead to mood disorders such as anxiety and depression and it appears that acyl ghrelin is important to prevent anxiety-like and depressive-like behaviors under stressed (18, 23, 24) and nonstressed conditions (25). These results highlight that acyl ghrelin release has significant implications for mood disorders (15) and in support of this, polymorphisms in the proghrelin gene are linked to major depression and anxiety disorders in humans (26, 27).

It is important to undertsand how ghrelin signals in the brain to regulate stress and anxiety and an often overlooked consideration is that ghrelin exists in the blood in two forms, acyl ghrelin and des-acyl ghrelin, with des-acyl ghrelin being the most abundant form in circulation (28). In ghrelin-secreting stomach cells, proghrelin is acylated in the endoplasmic reticulum by the enzyme ghrelin-O-acyltransferase (GOAT) and processed in the Golgi to acyl ghrelin. GOAT is colocalised with most ghrelin-secreting cells in the stomach and digestive tract (29–31). Des-acyl ghrelin is generated after acyl ghrelin is released into the circulation through the actions of endogenous esterases, including acyl protein thioesterase (APT1) among others (32–34). While acyl ghrelin acts at the growth hormone (GH) secretagogue receptor (GHSR), des-acyl ghrelin does not act at this receptor within a physiological range and evidence suggests des-acyl ghrelin acts on an alternative receptor (35).

There are a number of reports that des-acyl ghrelin has biological effects independent from the GHSR. For example, des-acyl ghrelin administration prevents cell death in cultured neurons exposed to oxygen and glucose deprivation in the presence of a GHSR antagonist (36, 37). In vivo, des-acyl and not acyl ghrelin administration yields a vasodilator response (38). In microglia exposed to amyloid-beta, des-acyl ghrelin attenuated the release of the proinflammatory cytokine IL-6, whereas acyl ghrelin had no effect (39). In addition, we have recently shown that des-acyl ghrelin specifically binds to and acts on a subset of arcuate nucleus (ARC) cells in a GHSR-independent manner and that it impairs the orexigenic actions of peripherally administered acyl ghrelin. Thus, des-acyl ghrelin directly targets ARC neurons and antagonizes the orexigenic effects of ghrelin (35).

We previously showed that acute stress increases anxiety-like behavior and HPA activity in Ghrelin KO mice (23). However, Ghrelin KO mice lack both acyl and des-acyl ghrelin and is unknown whether or not des-acyl ghrelin plays a significant role in the regulation of the stress axis or in anxiety-like behavior. Importantly, acyl ghrelin injections can be readily deacylated in vivo (9) and therefore it is possible that the effect of an acyl ghrelin injection on the HPA stress axis and/or anxiety-like behavior may be partially due to des-acyl ghrelin signaling. In this study we tested the effect of des-acyl ghrelin on the HPA axis and on anxiety-like behavior in des-acyl ghrelin treated mice lacking endogenous ghrelin, to avoid confounding endogenous ghrelin, and in GOAT KO mice that have no endogenous acyl ghrelin and high endogenous des-acyl ghrelin.

Materials and Methods

Animals

All experiments were conducted in compliance with the Monash University Animal Ethics Committee guidelines. Mice were kept at standard laboratory conditions with free access to food and water at 23°C in a 12-hour light/dark cycle unless otherwise stated. Male Ghrelin and GOAT KO mice on a C57/Bl6 background were obtained from Regeneron Pharmaceuticals (Tarrytown, NY) and bred (het x het; 1 male x 2 females) in the Monash Animal Services to generate WT and KO littermates. Genotyping was conducted according to previous reports (40). All mice were group-housed to prevent isolation stress. All injections were administered via an intraperitoneal route.

Des-acyl ghrelin injection

To examine the effect of des-acyl ghrelin on the HPA stress axis and anxiety-like behavior, Ghrelin KO mice (8–12 weeks) were treated chronically (7 days) with des-acyl ghrelin (1 mg/kg) at 9 am. This dose was chosen to achieve a plasma concentration of des-acyl ghrelin within the physiological range (Figure 1A&B), based on our unpublished data. The use of Ghrelin KO mice allows us to directly assess the impact of exogenous des-acyl ghrelin without endogenous acyl or des-acyl ghrelin acting as a confounding variable. To control for des-acyl ghrelin injections, Ghrelin KO were treated with saline. For behavioral experiments (n = 8–10), mice were tested under both nonstressed and acute restraint stressed conditions (15 minutes restraint in a ventilated Perspex tube, 3 cm in diameter, with an adjustable restraining length to a maximum of 10 cm). Behavioral analysis started at 11 am 2 hours after the last injection of des-acyl ghrelin. Additional cohorts were used for 1) measurements of plasma corticosterone under nonstressed and stressed conditions and 2) neuronal activation under nonstressed and stressed conditions (n = 6–10).

Behavioral testing

Elevated plus maze. At 10–12 wks of age, mice (n = 8–10) were tested for 7 minutes in the elevated plus maze test for anxiety in a novel environment, as described previously (41–44).

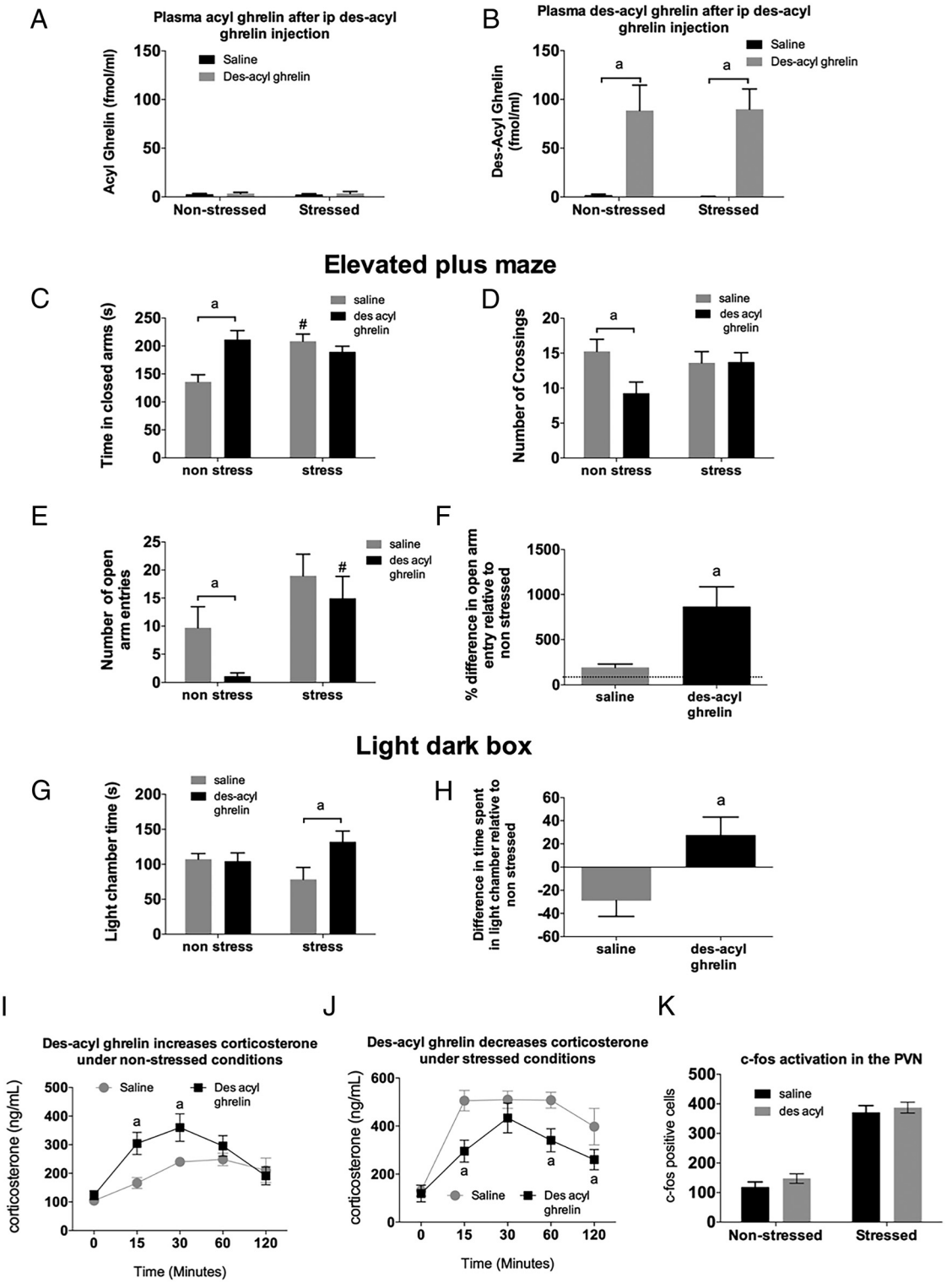

Figure 1. Des-acyl ghrelin treatment in Ghrelin KO mice affects anxiety-like behavior and influences plasma corticosterone under nonstressed and stressed conditions. No plasma acyl ghrelin was observed after des-acyl ghrelin treatment (A), whereas plasma des-acyl ghrelin (B) was significantly elevated compared to saline controls. Elevated plus maze (EPM) analysis of anxiety-like behavior showed that des-acyl ghrelin treatment increased time spent in the closed arms (C) in nonstressed mice compared to saline-treated controls. The time spent in the closed arm was significantly increased in saline treated controls but not in des-acyl ghrelin treated mice. The number of crosses were reduced in des-acyl ghrelin treated mice in non stressed conditions however no difference relative to saline controls was observed under stressed conditions (D). Open arm entries were lower in nonstressed des-acyl ghrelin treated mice however open arm entries were restored to saline controls in stressed des-acyl ghrelin treated mice (1E&F). Light dark box analysis analysis showed that des-acyl ghrelin elevated time spent in the light chamber only after stress (1G&H). Des-acyl ghrelin treatment under nonstressed (I) and stressed conditions (J) significantly elevated and reduced plasma corticosterone respectively. K, No

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 using Ethovision software (Noldus Information Technology; Wageningen, The Netherlands). After one week recovery from the initial nonstressed test, the mice were given 15 minutes restraint stress and tested again immediately poststress.

Light / dark box test for anxiety. In separate groups of mice were tested in the light / dark box test for anxiety (45). Each mouse was placed in an enclosed (dark) arena (22 × 30 x 25 cm), filmed and later scored for the time spent exploring the high light arena (22 × 30 x 25 cm) in a 5 minutes trial. The mice underwent a basal trial followed 7 days later by a stress trial as described above.

Plasma corticosterone in response to stress. On the day of experimentation mice (n = 6–10/group) were brought into the testing room at 7 am and allowed 4 hours to acclimatize to the room before 15 minutes restraint stress (or no stress for controls). Tail vein blood samples (<10ul) were collected at 0, 15, 30, 60, 120 minutes after restraint stress. A standard corticosterone enzyme immunoassay (EIA) kit (Abnova Corp., Taipei, Taiwan) was used to assess plasma corticosterone according to the manufacturer's instructions. This assay requires only 1μl of plasma to accurately measure plasma corticosterone. All samples were assayed in duplicate in the same plate. In order to accurately measure plasma acyl and des acyl ghrelin, blood collection tubes were treated with Pefabloc SC (Roche Applied Science, Mannheim, Germany) to achieve a final concentration of 0.5 mg/ml. Blood was centrifuged and the collected plasma was acidified with HCl (final concentration 0.05N). Plasma ghrelin levels were determined using Active Ghrelin or Des-acyl Ghrelin Enzyme-Linked Immunoassay Kits (Mitsubishi Chemical Medicine, Tokyo, Japan). Active and des-acyl ghrelin were measured according to kit instructions. The interassay variability for all ELISAs was less than 10% coefficient of variation.

Neuronal activation in response to stress. Mice (n = 6–10) were allowed to acclimatise for 4 hours in the experimental room before testing. 60 minutes after stress-onset, mice were anesthetized and perfused with PBS followed by 4% paraformaldehyde in PBS (4°C, pH 7.4). Brains were removed and postfixed for 24 hours in the same fixative before being cryoprotected with 20% sucrose in PBS (4°C). Forebrains were subsequently cut using a cryostat into 40 μm coronal sections and 4 sections per animal (spaced 8 sections apart to cover the PVN) were analyzed for cfos expression. Neuronal activation was assessed on the basis of positive c-fos-immunoreactivity (24 hours, 4°C; 1:10 000; rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA). c-fos-positive cells were counted blind in regions of interest (46, 47).

Assessment of CRH / c-fos colocalization. As previously described in detail (Cabral et al, 2012). Briefly, sections were pretreated with H2O2, treated with blocking solution and incubated with anti-c-fos antibody (Calbiochem, cat. PC38, 1:15,000) for 2 days at 4°C. Then, sections were incubated with biotinylated antirabbit antibody (Vector Laboratories, cat. BA-1000, 1:1,500) for 1 hour and with Vectastain Elite ABC kit (Vector Laboratories, cat. PK-6200) according to manufacturer's protocols. Finally, visible signal was developed with diaminobenzidine (DAB)/Nickel solution (Sigma-Aldrich, cat. 32 750), which generated a purple-black precipitate. Double c-fos and CRH immunostaining was performed on serial sets of sections containing the PVN. In this case, c-fos immunostained sections were then incubated with a rabbit anti-CRH antibody (1:2,000) for 48 hours, and then sequentially incubated with the secondary antibody and the Vectastain Elite ABC kit, as detailed above. Finally, visible signal was developed with DAB solution without nickel, which generates a brown precipitate. Sections were sequentially mounted, and bright-field images were acquired with a DS-Ri1 Nikon digital camera. To determine the total number c-fos-immunoreactive (IR) cells in the ARC and the PVN, cells containing distinct nuclear black precipitate were quantified between bregma –1.58 and –1.94 mm for the ARC, and between bregma –0.70 and –0.94 mm for the PVN. Anatomical limits of each brain region were identified using a mouse brain atlas (Paxinos and Franklin, 2001). c-fos data were expressed as total c-fos-IR cells per ARC/side coronal section. To estimate the amount of CRH-IR cells positive for c-fos, all CRH-IR cells positive and negative for c-fos were counted in the PVN. The results were expressed as a percentage, which represents CRH-IR neurons positive for c-fos compared to the total number of CRH-IR neurons. Data were corrected for double counting, according to the method of Abercrombie (Abercrombie, 1946) where the ratio of the actual number of neurons to the observed number is represented by T/T+h where T=section thickness, and h=the mean diameter of the neuron along the axis perpendicular to the plane of section. The mean diameter of the neurons was determined with the image analysis software program ImageJ (NIH). Blind quantitative analysis was performed in one series per animal and by two observers.

CRH In situ hybridization

The RNA probe was generated using the full-length rat CRH cDNA sequence (kindly provided by the late W.W. Vale), which is > 95% homologous with the mouse and binds mouse CRH mRNA with high affinity (48), and labeled with DIG-11-UTP using the labeling kit from Roche Diagnostics GmbH (Mannheim, Germany), according to manufacturer's instructions. In situ hybridization incubation started with selecting tissue sections containing the regions of interest. The rest of the protocol was done using the free-floating method, using sterile Costar 6-well plates with Netwell inserts (Corning) and RNase-free solutions and glassware. The sections were rinsed 5 times in 0.1M PBS to wash out the cryoprotectant and were subjected to additional fixation in 0.1M borax-buffered 4% paraformaldehyde (pH 9.5) at 4°C for 30 minutes. The sections were then rinsed three times with 0.1M PBS at room temperature, followed by a 10 minutes incubation with proteinase K medium (0.1M Tris/HCl, 0.05M EDTA, 0.01 mg/mL proteinase K, pH 8.0) at 37°C. After rinsing in distilled water, acetylation was performed with

Legend to Figure 1 Continued. . .
difference in c-fos activation in the PVN was detected between saline and des-acyl ghrelin treated mice. All data are presented as mean +/- sem, n = 8–10/group. a, significant with respect to treatment; # significant with respect to stress within the same treatment group.

0.25% acetic acid anhydride in 0.1M triethanolamine (TEA) buffer (pH 8.0) at room temperature for 10 minutes, and rinsing in 2 × standard saline citrate (SSC) for 5 minutes. Hybridization medium, consisting of 50% deionized formamide, 0.3M NaCl, 0.001M EDTA, 1 × Denhardt's solution, 10% dextran sulfate, and 0.5 mg/mL tRNA (Roche), was placed together with the mRNA-DIG probe in a water bath at 80°C for 5 minutes and then on ice for 5 minutes. The 2 × SSC buffer was replaced by the hybridization solution (1 mL in each vial) for overnight incubation in a water bath at 58°C. After incubation, sections were rinsed three times with 4 × SSC buffer at room temperature, followed by incubation in preheated RNase medium (0.5M NaCl, 0.01M Tris/HCl, 0.001M EDTA, pH 8.0, and 0.01 mg/mL RNAse A (Roche) that had been added just before the start of the incubation) for 30 minutes at 37°C, and by stringent washing steps with decreasing concentrations of SSC (2 ×, 1 ×, 0.5 ×, 10 minutes per step, room temperature) with a final rinsing in 0.1 × SSC for 30 minutes at 58°C. The alkaline phosphatase method with NBT/BCIP as substrate was used for the detection of the DIG label. Briefly, after rinsing two times 10 minutes with buffer A (0.1M Tris/HCl, 0.15M NaCl, pH 7.5), sections were preincubated in buffer A containing 0.5% blocking agent (Roche) for 60 minutes, followed by a 3 hours incubation at room temperature with sheep anti-DIG-AP (Roche; 1:5000) antibody in buffer A containing 0.5% blocking agent. Then, sections were rinsed two times for 10 minutes in buffer A, followed by 2 × 5 minutes in buffer B (0.1M Tris/HCl, 0.15M NaCl, 0.05M $MgCl_2$, pH 9.5). After overnight incubation at room temperature in NBT/BCIP medium (10 mL buffer B, 2.4 mg levamisole, 175 μL NBT/BCIP mixture (Roche)) in a light-tight box, the reaction was stopped by placing the sections in buffer C (0.1M Tris/HCl, 0.01M EDTA, pH 8.0). After rinsing twice for 5 minutes, sections were mounted on gelatin-coated microscopy slides, dried overnight at 37°C, and covered with a standard cover glass using Kaiser's glycerol gelatin (Boom BV, Meppel, the Netherlands). Slides were allowed to dry at room temperature overnight, before storage in a light-tight box until needed. For Image analysis the in situ hybridization signal was determined by measuring the intensity of the CRH-positive neurons. For this, the staining intensity of each stained neuron was measured using the Point tool of ImageJ in the most stained part of the neuron. This value was subtracted from the intensity of the background to obtain the SSD. In addition, we noted the number of stained neurons per region of interest. For in situ hybridization experiments, three sections per region of interest per animal were evaluated and the mean per animal recorded.

Statistical Analysis

All data are represented as mean ± standard error of the mean (SEM). Two-Way ANOVAs with Fisher LSD post hoc tests were used to determine statistical significance between treatment and genotype. A two-tailed Student's unpaired *t* test was used when comparing genotype only. $P < .05$ was considered statistically significant.

Results

Des-acyl ghrelin treatment

Daily des-acyl ghrelin injections for 7 days to ghrelin KO mice resulted in significantly detectable des-acyl ghrelin in the plasma at 90 minutes after the final injection. Levels were within the physiological range of wild type endogenous des-acyl ghrelin. No plasma acyl ghrelin was detectable (Figure 1A&B; ANOVA - interaction F (1, 27) = 0.007, $P = .9308$; stress status F (1, 27) = 1.9e-005, $P = .9966$; treatment F (1, 27) = 26.48, $P < .0001$). These results demonstrate des-acyl ghrelin was not converted to acyl ghrelin and the data collected are specific to the actions of des-acyl ghrelin.

Anxiety-like behaviors

To investigate the role of des-acyl ghrelin in stress and anxiety in the absence of confounding circulating acyl ghrelin, we injected des-acyl ghrelin daily for 7 days in Ghrelin KO mice and tested anxiety-like behaviors and HPA activation in response to acute restraint stress. Under nonstressed conditions, des-acyl injections significantly increased anxiety-like behavior in the EPM. This includes increasing the time spent in closed arm (Figure 1C; ANOVA - interaction F (1, 27) = 12.26, $P = .00016$; stress status F (1, 27) =3.570, $P = .0696$; treatment F (1, 27) = 4.424, $P < .0449$), reducing the number of crosses across the center point (Figure 1D; ANOVA - interaction F (1, 27) = 3.632, $P = .0674$; stress status F (1, 27) =0.7896, $P = .3821$; treatment F (1, 27) = 3.422, $P < .075$) and decreasing the number of open arm entries (Figure 1E; ANOVA - interaction F (1, 27) = 0.4784, $P = .4784$; stress status F (1, 27) =12.16, $P = .0017$; treatment F (1, 27) = 3.619, $P < .0678$). After acute restraint stress, saline-treated Ghrelin KO mice spent more time in closed arms compared to nonstressed saline-treated mice, however des-acyl ghrelin treatment did not increase closed arm time in stressed relative to nonstressed mice (Figure 1C). Des-acyl ghrelin treatment under acute restraint stress also normalized the number of EPM crosses during the trial back to saline-treated levels (Figure 1D). Des-acyl ghrelin significantly increased the number of open arm entries under acute stress relative to nonstressed conditions, similar to saline-treated stressed mice (Figure 1E). The greater effect of des-acyl ghrelin, compared to saline, to increase the number of open arm entries after stress is shown in Figure 1F.

To strengthen our analysis of anxiety-like behavior, we also performed light / dark box analysis. In nonstressed conditions des-acyl ghrelin had no effect on time spent in the light or dark chamber (Figure 1G; ANOVA - interaction F (1, 32) = 4.582, $P = .0400$; stress status F (1, 32) =0.00226, $P = .9623$; treatment F (1, 32) = 3.704, $P < .0632$). After acute restraint stress des-acyl ghrelin treatment increased the time spent in the light chamber (Figure 1G). Relative to nonstressed conditions, des-acyl ghrelin increased the time spent in the light chamber after stress

compared to saline treated mice (Fig H). Collectively the data from both the EPM and light / dark box show that des-acyl ghrelin increases anxiogenic behaviors under nonstressed conditions but has anxiolytic effects after acute restraint stress.

HPA axis activation

Under nonstressed conditions, des-acyl ghrelin increased plasma corticosterone (Figure 1I; ANOVA - interaction F (4, 47) = 2.26, P = .0772; time F (4, 47) =10.37, $P < .0001$; treatment F (1, 47) = 9.545, P = .0034), whereas des-acyl ghrelin significantly reduced corticosterone 15, 60 and 120 minutes after acute restraint stress compared to saline controls (Figure 1J; ANOVA - interaction F (4, 47) = 1.424, P = .2414; time F (4, 47) =16.58, $P < .0001$; treatment F (1, 47) = 17.25, P = .0001). These observations provide a biological substrate that may underlie the differences in anxiety-like behavior reported above. However despite the differences in plasma corticosterone and anxiety-like behavior we did not observe any treatment differences in numbers of c-fos-positive paraventricular nucleus of the hypothalamus (PVN) cells in either nonstressed or stressed conditions (Figure 1K), although stress itself resulted in a significant increase in PVN c-fos-positive cells (ANOVA - interaction F (1, 21) = 0.0188, P = .7451; stress status F (1, 21) =167.0, $P < .0001$; treatment F (1, 47) = 1.410, P = .2497).

GOAT KO mouse studies

Anxiety-like behaviors

GOAT is the enzyme that acylates proghrelin in ghrelin-secreting cells. Deletion of GOAT in mice results in higher levels of des-acyl ghrelin throughout the entire span with no measurable acyl ghrelin (49). To reinforce the studies on des-acyl ghrelin treatment in Figure 1 we tested whether chronic des-acyl ghrelin elevation from GOAT deletion affected anxiety-like behavior. Findings from the EPM revealed that restraint stress significantly increased time spent in closed arms and decreased the number of center crossings compared to nonstressed conditions (main effect), although no genotypic difference were observed (Figure 2A, ANOVA - interaction F (1, 28) = 0.0187, P = .892; stress status F (1, 28) =14.28, P = .0008; genotype F (1, 28) = 0.3632, P = .5516; Figure 2B ANOVA - interaction F (1, 28) = 0.1575, P = .6947; stress status F (1, 28) =31.21, $P < .0001$; genotype F (1, 28) = 1.276, P = .2690). However, the number of open arm entries highlighted a significant main effect of genotype with GOAT KO showing fewer open arm entries (Figure 2C; ANOVA - interaction F (1, 28) = 4.270, P = .0489; stress status F (1, 28) =0.0903, P = .7661; genotype F (1, 28) = 25.70, $P < .0001$). Moreover, stress significantly reduced open arm entries in GOAT KO relative to WT mice (Figure 2C) and the percent difference in open arm entries relative to nonstressed levels was significantly lower in GOAT KO mice (Figure 2D).

Light / dark box analysis showed that GOAT KO mice spent significantly less time in the light chamber (main effect of genotype; Figure 2E; ANOVA - interaction F (1, 27) = 16.37, P = .0004; stress status F (1, 27) =0.3649, P = .5508; genotype F (1, 27) = 21.36, $P < .0001$). Light chamber time was significantly reduced in stressed GOAT KO relative to stressed GOAT WT mice and the percent difference in light chamber time relative to nonstressed conditions was significantly lower in GOAT KO mice (Figure 2E, F). These results illustrate that GOAT KO display greater anxiety-like behavior particularly under stressed conditions.

HPA axis activation

In response to acute restraint stress or an ACTH injection, we observed no genotype differences plasma corticosterone concentrations (Figure 2G-H), showing that the differences in stress-induced anxiety behaviors were not due to impaired HPA activation (Figure 2G, ANOVA - interaction F (4, 64) = 0.8361, P = .5104; time F (4, 40) =21.14, $P < .0001$; genotype F (1, 40) = 0.1092, P = .7428; Figure 2H, ANOVA - interaction F (4, 64) = 1.415, P = .2391; time F (4, 64) =24.46, $P < .0001$; genotype F (1, 64) = 0.7312, P = .3957).

We counted c-fos-positive neurons in the PVN in stressed and nonstressed conditions. Stress significantly increased numbers of c-fos-positive cells (significant main effect) in the medial parvocellular (MP), dorsal parvocellular (DP), magnocellular (MG) and total PVN, with significantly more c-fos-positive cells in the MP and MG in GOAT KO relative to GOAT WT mice (Figure 3A-D; 3A, ANOVA interaction F (1, 24) = 2.212, P = .1500; stress status F (1, 24) =84.23, $P < .0001$; genotype F (1, 24) = 9.687, P = .0047; 3B, ANOVA interaction F (1, 24) = 2.958, P = .0983; stress status F (1, 24) =21.10, P = .0001; genotype F (1, 24) = 1.437, P = .2424; 3C ANOVA interaction F (1, 24) = 0.000862, P = .9768; stress status F (1, 24) =23.73, $P < .0001$; genotype F (1, 24) = 2.297, P = .1445; 3D ANOVA interaction F (1, 24) = 3.398, P = .0794; stress status F (1, 24) =35.79, $P < .0001$; genotype F (1, 24) = 2.307, P = .1437).

Dual label immunohistochemistry revealed more c-fos-positive/CRH neurons in the MP but not the DP PVN in GOAT KO relative to GOAT WT mice (Figure 3G-H; 3G, ANOVA interaction F (1, 12) = 5.371, P = .0389; stress status F (1, 24) =311.6, $P < .0001$; genotype F (1, 24) = 1.477, P = .2477; 3H, ANOVA interaction F (1, 12) =

0.2851, $P = .6031$; stress status F (1, 12) =57.63, $P < .0001$; genotype F (1, 12) = 1.501e-013, $P > .9999$). In previous studies we identified ghrelin KO mice have significantly more c-fos-positive cells in the medial amygdala (MeA) and centrally projecting Edinger Westphal nucleus (EWcp) under both stress and nonstressed conditions (23). To examine whether GOAT WT and KO mice displayed similar differences, we examined neuronal activation in these regions. While we observed a significant main effect of stress to increase c-fos-positive cell numbers in the MeA and EWcp, there were no differences between the genotypes under stressed conditions (Figure 3E, F; 3E, ANOVA interaction F (1, 23) = 2.989, $P = .0972$; stress status F (1, 23) =50.96, $P < .0001$; genotype F (1, 23) = 2.604, $P = .1203$; 3H, ANOVA interaction F (1, 23) = 0.07923, $P = .9298$; stress status F (1, 23) =45.04, $P < .0001$; genotype F (1, 23) = 0.007923, $P > .9298$). However, we observed fewer c-fos-positive neurons in the MeA of GOAT KO mice, compared to GOAT WT mice, in nonstressed conditions.

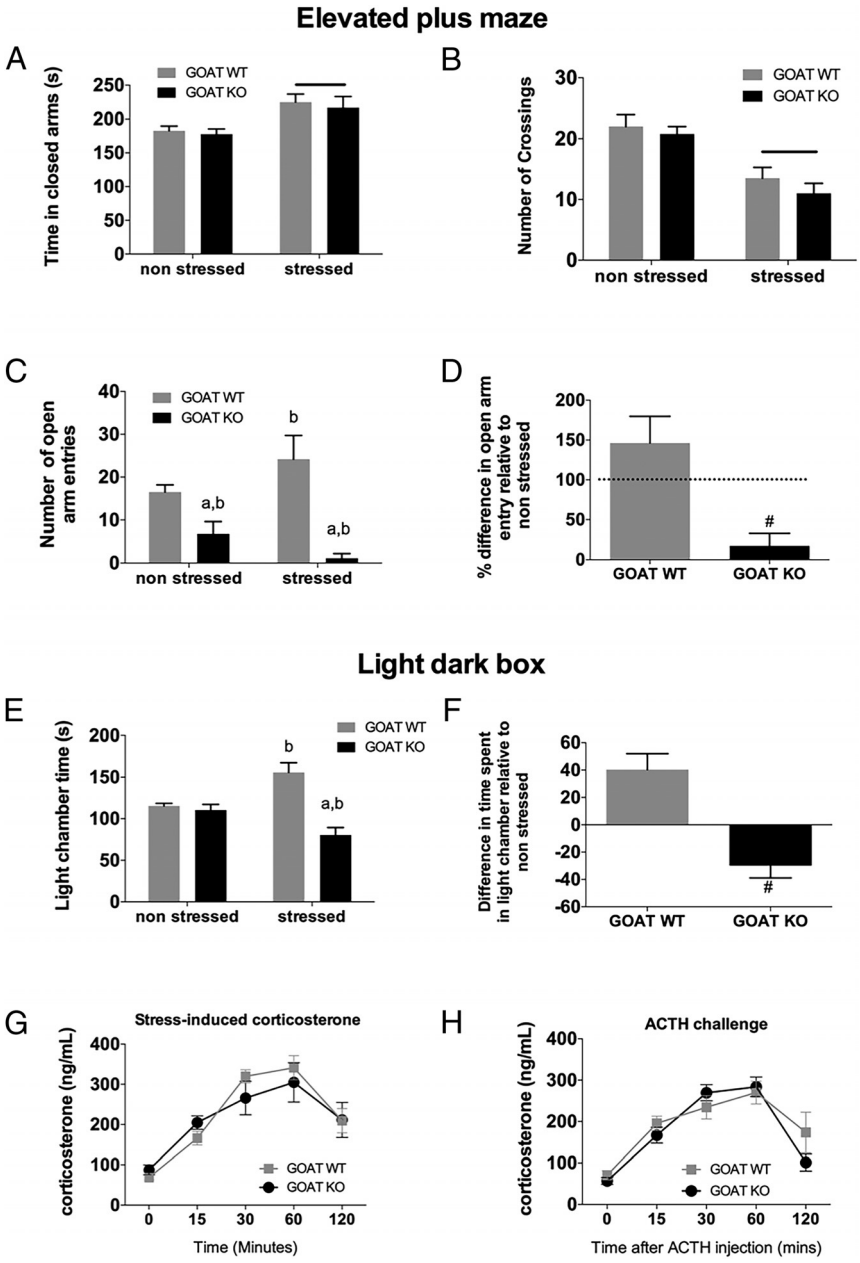

Figure 2. GOAT KO mice exhibit increased anxiety-like behavior without influencing plasma corticosterone. Elevated plus maze analysis indicated that stress significantly increase the time spent in the closed arms (A) and decreased the number of crossings (B) in both GOAT WT and KO mice with no differences between genotypes The lines above the stressed groups in A&B represent significant main effects. GOAT KO mice showed fewer open arm entries in both nonstressed and stressed conditions (C) and the relative difference in response to stress between the genotypes is shown in D. In response to stress, GOAT KO mice spend significantly less time in the light chamber compared to GOAT WT mice. Relative to the nonstressed conditions GOAT KO spend significantly less time in the light chamber when compared to WT mice (F). No differences in stress- or ACTH-induced plasma corticosterone concentrations were observed (G,H). All data are presented as mean +/- sem, n = 10/group; two way ANOVA. a, significant with respect to genotype within the same treatment (non stressed vs stressed) group. b, significant with respect to non stressed vs stressed within the same genotype. #, significant with respect to GOAT WT.

In light of these findings we further investigated the CRH mRNA levels in GOAT WT and KO mice under stressed and nonstressed conditions. In situ hybridization studies showed that stress significantly increased CRH mRNA specific signal density (SSD) in the bed nucleus of the stria terminalis (BNST) and PVN (significant main effect) but not the central amygdala (CeA). However, we observed no genotype differences in PVN, BNST or CeA CRH mRNA expression between GOAT WT and KO mice (Figure 4A-C; 4A, ANOVA interaction F (1, 15) = 0.09295, $P = .7647$; stress status F (1, 15) =16.59, $P = .0010$; genotype F (1, 15) = 0.5515, $P = .4692$; 4B, ANOVA interaction F (1, 15) = 0.3104, $P = .5856$; stress status F (1, 15) =16.15, $P = .0011$; genotype F (1, 15) =

Table 1. Antibody Table

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
c-*fos*		anti-cfos	SC 52, Santa Cruz Biotechnology, Santa Cruz, CA	rabbit poly clonal	1 in 1000; 1 in 10 000
c-*fos*		anti-cfos	Calbiochem, cat. PC38	rabbit poly clonal	1:15 000
CRH		rat anti-CRH	Advaced Targeting System; AB-02	rabbit polyclonal	1000
Digoxigenin		anti-digoxigenin	Roche, #11093274910	sheep polyclonal	1 in 5000

0.2016, $P = .6599$). Neither stress nor genotype had an effect on CRH neuronal number in the BNST, PVN or CeA, suggesting no defects in the CRH system after the deletion of GOAT.

Discussion

Ghrelin exists in two forms in circulation, acyl ghrelin and des-acyl ghrelin, both of which have distinct and fundamental roles in a variety of physiological functions. Despite this fact, a large proportion of papers simply measure and refer to plasma "ghrelin" without specifying the acylation status. Recent data, including the present study, now show that acyl and des-acyl ghrelin's distinct roles include neuroprotective function (8, 9, 36, 37), metabolism (50–53), cerebrovascular function (38), inflammation (39), endocrine responses (2), food intake regulation (35), and now stress-responsiveness and anxiety. It is therefore critical to assess and state the acylation status of

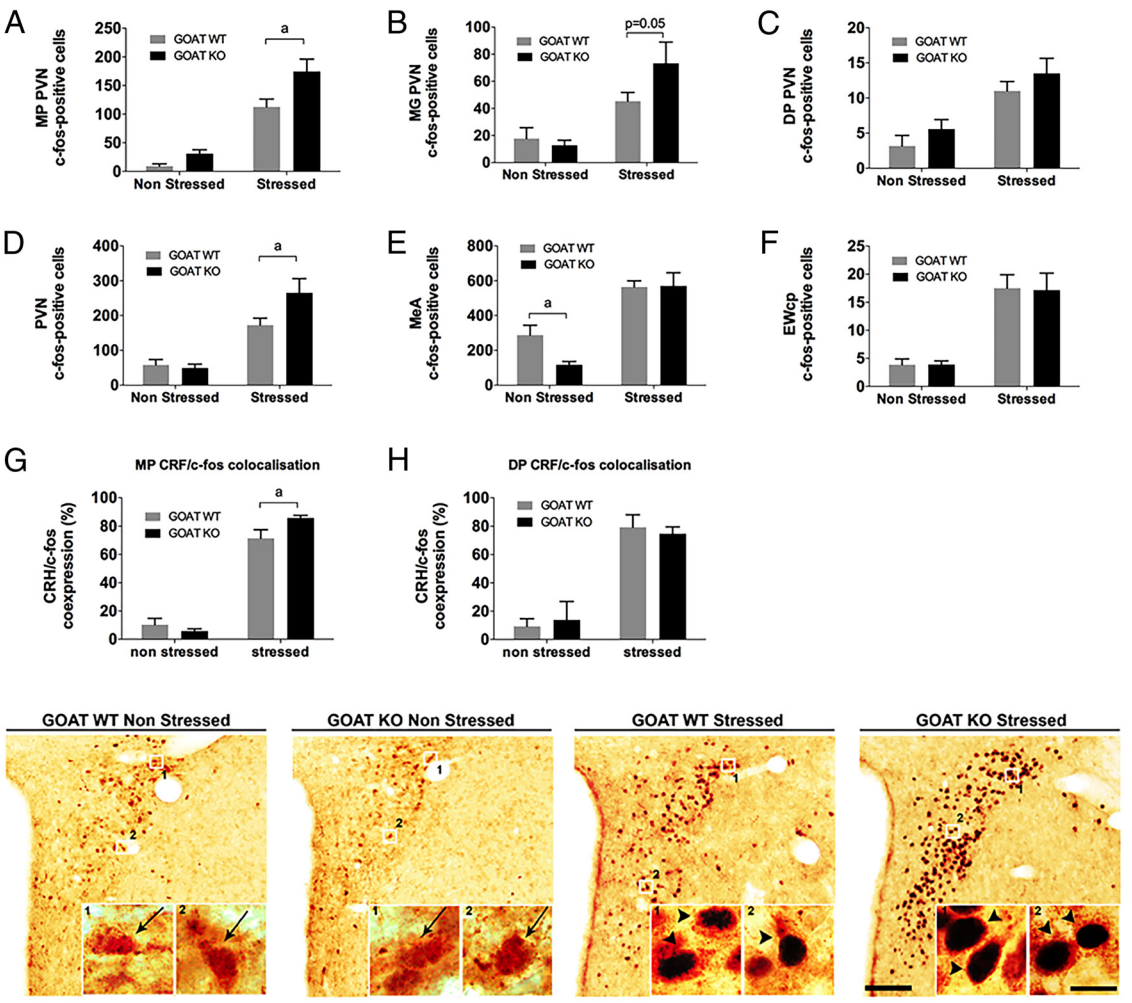

Figure 3. GOAT KO mice show greater c-fos activation in the PVN. Stress increased the number of c-fos-positive neurons in the medial parvocellular (MP) PVN, the magnocellular (MG) PVN, the dorsal parvocellular (DP) PVN, the total PVN, the medial amygdala (MeA) and the edinger-westphal nucleus (EWpc) compared to nonstressed conditions (A-F). Moreover, the c-fos-activation in response to stress was significantly higher in GOAT KO in the MP (A), MG (B) and total PVN (D). No genotypic differences were observed in the DP PVN (C), MeA (E) and EWpc (F). There was also greater CRH/c-fos coexpression in the MP PVN (G) but not the DP PVN. Representative images showing CRH/c-fos coexpression in the PVN in both stressed and non stressed conditions. Data are presented as mean +/- sem, n = 4–7 per group, two-way ANOVA.

plasma ghrelin in all studies. Furthermore, we believe that all future studies should specifically refer to plasma ghrelin as either acyl or des-acyl ghrelin. This is becoming more important since acyl ghrelin and des-acyl ghrelin can have concordant or discordant effects on physiology and behavior.

A large number of recent studies suggest that acyl ghrelin plays an important role in the activation of the HPA stress axis and to control anxiety and mood-related disturbances (15, 16, 18, 23, 25, 54, 55). However, none of the studies has examined the role of des-acyl ghrelin on the activation of the HPA axis and anxiety-like behaviors. Here, we specifically addressed whether or not des-acyl ghrelin regulates activation of the HPA axis and anxiety-behavior in two different models; GOAT WT and KO mice and Ghrelin KO mice that we treated with saline, des-acyl for 7 days. The use of Ghrelin KO mice allows us to directly assess the impact of exogenous des-acyl ghrelin without endogenous acyl or des-acyl ghrelin acting as a confounding variable. The use of GOAT KO mice allows us to assess chronically high levels of endogenous des-acyl ghrelin, as deletion of the GOAT enzyme prevents proghrelin from being acylated. One limitation with these approaches is that global deletion of ghrelin or GOAT may have unintended effects on the development of circuits involved in the HPA stress. Studies with Ghrelin KO mice and acyl ghrelin injections show an important underappreciated role on the development of hypothalamic feeding circuits (56), similar effects may affect hypothalamic stress circuits although this possibility has not been addressed.

Our results show that both des-acyl ghrelin treatment in Ghrelin KO mice and the absence of acyl ghrelin in GOAT KO mice affected activation of the HPA axis and subsequent anxiety-like behaviors, however the different models did not produce cohesive findings in aspects of HPA activation and anxiety-like behavior, suggesting that the high levels of des-acyl ghrelin are not the sole reason for the anxiety-like behavior in GOAT KO mice. In terms of behavior, des-acyl ghrelin treatment in Ghrelin KO mice and the absence of acyl ghrelin in GOAT KO mice lead to an anxiogenic phenotype under nonstressed conditions, which was further exacerbated by stress in GOAT KO mice but reversed in des-acyl ghrelin treated mice. That fact that des-acyl ghrelin treatment and GOAT KO show an anxiogenic phenotype under non stressed conditions is consistent with most studies using acyl ghrelin (16, 23, 57, 58), although this is not always observed (25). Moreover, under stressed conditions treatment of des-acyl ghrelin, similar to the actions of acyl ghrelin, reduced anxiety (15, 18, 25). These observations suggest that both acyl ghrelin and des-acyl are involved in the proposed dual role of ghrelin peptides in anxiety-like behavior, with the presence of an acute or chronic stressor the switch that flips between anxiogenic and anxiolytic states. Physical and psychological stressors increase acyl ghrelin concurrently with glucocorticoid release (15) and acyl ghrelin is readily converted in the plasma to des-acyl ghrelin via the actions

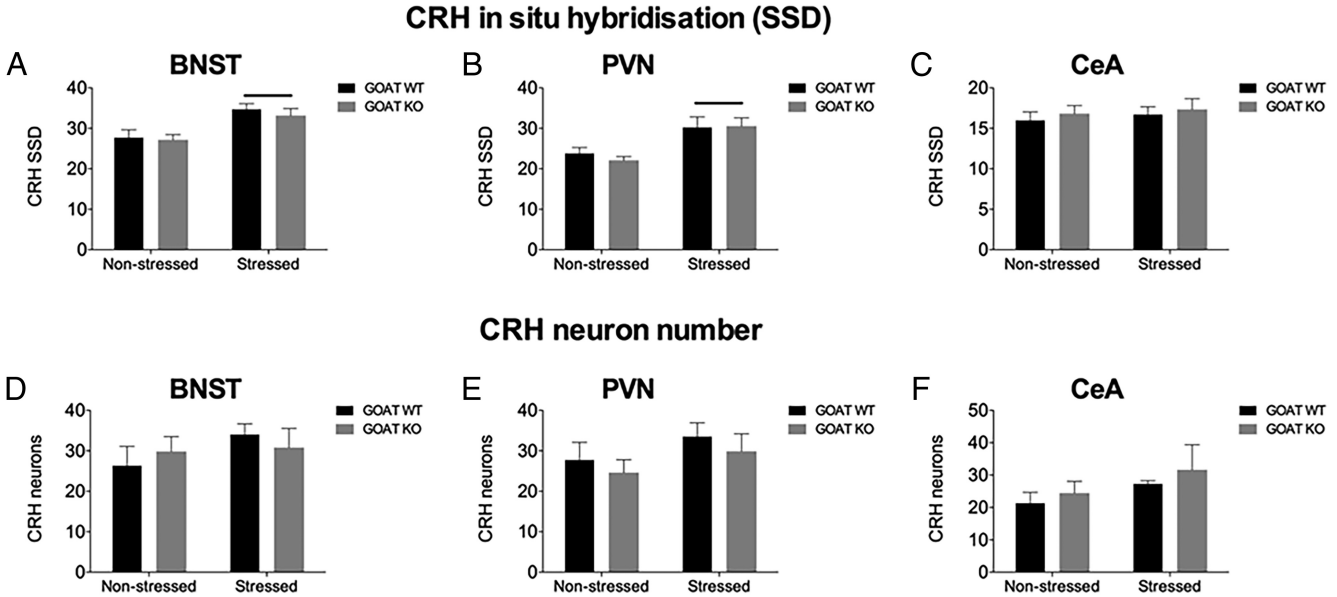

Figure 4. GOAT deletion does not affect CRH mRNA expression or CRH cell number. Although stress increases CRH mRNA expression (significant main effect) in the BNST (A) and PVN (B), no differences between GOAT WT and KO mice were observed. Neither stress nor genotype affected CRH mRNA in the Central Amygdala (CeA; C). Neither stress nor genotype affected the number of CRH cells in the BNST (D), PVN (E) or CeA (F). Data are presented as mean +/- sem, n = 4–6 per group, two-way ANOVA.

of endogenous esterases (32–34). Thus, it is possible that endogenous conversion of acyl ghrelin to des-acyl ghrelin influences anxiety-like behavior. Further, the observed effects of exogenous acyl ghrelin on anxiety-like behavior may be partially caused by des-acyl ghrelin since deacylation readily occurs in the plasma after exogenous acyl ghrelin injection (9). Conversely, we show in this study that exogenous des-acyl ghrelin cannot be converted to acyl ghrelin in the plasma.

Consistent with a dual role for des-acyl ghrelin on anxiety-like behavior, des-acyl ghrelin, in the absence of acyl ghrelin, significantly increased plasma corticosterone under nonstressed conditions but decreased plasma corticosterone under stressed conditions. We have recently shown that central administration of des acyl ghrelin increases c-fos levels in the PVN of wild-type mice. However, the changes in plasma corticosterone levels detected in the current study did not correlated with c-fos activation in the PVN, suggesting that the effect of des-acyl ghrelin on the HPA axis may be regulated through additional brain sites.

Studies indicate that des-acyl ghrelin interacts with CRH receptor 2 but not CRH receptor 1 subtypes (59) and CRH receptor 2 activation reduces anxiety-like behavior independent from the HPA axis (60, 61). Thus it may be that acyl ghrelin acts via CRH in the PVN (54), whereas des-acyl ghrelin acts via CRH receptor 2 neurons in other regions of the brain.

Unlike with des-acyl ghrelin treatment in Ghrelin KO, GOAT KO mice showed exacerbated anxiety-like behavior in response to stress. It is important to note that GOAT KO mice cannot synthesize acyl ghrelin and have high levels of des-acyl ghrelin exclusively. Similarly, our des-acyl ghrelin treatment was in Ghrelin KO mice and therefore both models had no acyl ghrelin that could confound the interpretation of the results. GOAT is an membrane-bound octanoic acyltransferase and acylation of proghrelin is dependent upon GOAT (29, 31) and although GOAT is coexpressed with ghrelin-secreting cells in the gut, GOAT is expressed in numerous other tissues not known to produce ghrelin (29–31, 62, 63). It is therefore possible that GOAT KO mice may have additional unidentified physiological changes, unrelated to ghrelin acylation, that influence anxiety-like behavior.

Neither stress-induced nor ACTH-induced plasma corticosterone was different between genotypes; however, restraint stress significantly increased the number of c-fos-positive neurons in the MP PVN in GOAT KO relative to WT mice, with greater numbers CRH/c-fos-positive neurons in the same region. This suggests the feedback actions of glucocorticoids to reduce c-fos activation in the PVN were suppressed in GOAT KO mice. The lack of difference in stress-induced and ACTH-induced plasma corticosterone suggests normal ACTH release and action at the adrenal glands. Interestingly, a deficit in ACTH release from the pituitary and action at the adrenal gland was seen previously Ghrelin KO mice (23). However, the nature of c-fos-immunoreactivity means it is impossible to ascribe a direct action at the nucleus of interest, as it could be a result of neural inputs into the PVN from other regions. Further characterization the CRH system with in situ hybridization and immunohistochemistry revealed that stress did not differentially effect mRNA gene expression and CRH cell number in the BNST, PVN or CeA. Thus, similar to acyl ghrelin (54), the activation of CRH neurons in the PVN is presumably due to indirect activation from other, unknown sites. Interestingly, we observed reduced stress-induced neuronal activation in the MeA of nonstressed GOAT KO mice relative to WT mice. This region contains GHSRs (64) and has been previously demonstrated to show differential activation of c-fos in Ghrelin KO mice (23).

It is worth noting that other effects could account for the differences between des-acyl treatment in Ghrelin KOs and the effects seen in GOAT KO mice. First, GOAT KO mice have chronically elevated plasma des-acyl ghrelin throughout their entire lifespan, whereas des-acyl ghrelin treatment to Ghrelin KO mice only produced high levels of ghrelin during the 7-day treatment period. Second, des-acyl ghrelin treatment was performed in the Ghrelin KO mice and although this was an important control to match the lack of acyl ghrelin in GOAT KO mice, Ghrelin KO mice have deficits in a number of physiological functions, including stress and neuroprotection (9). Third, an injection of des-acyl ghrelin causes a bolus dose that does not match the relatively high and stable des-acyl ghrelin levels seen in GOAT KO mice.

Our results suggest that des-acyl ghrelin treatment in Ghrelin KOs and GOAT deletion have differential effects on the HPA axis and anxiety-like behavior suggesting that the anxiogenic actions of GOAT deletion are not related to high plasma des-acyl ghrelin. These results are in line with Rucinski et al (65) where they showed that inhibition of GOAT acutely suppressed CRH mRNA expression and plasma corticosterone concentrations but did not affect plasma acyl of des-acyl ghrelin concentrations. Similar to acyl ghrelin treatment, des-acyl ghrelin produces an anxiogenic effect under nonstressed conditions but this switches to an anxiolytic effect under stressed. Des-acyl ghrelin given to Ghrelin KOs influences plasma corticosterone under both nonstressed and stressed conditions although c-fos activation in the PVN is not different. These results suggest that des-acyl ghrelin presumably influences the HPA axis at peripheral sites and the feedback actions

of glucocorticoids act on brain regions outside of the PVN. By contrast, GOAT KO are anxious under both non-stressed and stressed conditions although this is not due to corticosterone release from the adrenals but rather from impaired feedback actions in the PVN, as assessed by c-fos activation. These studies further our understanding of the ghrelin/GOAT system on anxiety and stress reactivity.

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