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A ghrelin–growth hormone axis drives stress-induced vulnerability to enhanced fear

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Hormones in the hypothalamus–pituitary–adrenal (HPA) axis mediate many of the bodily responses to stressors, yet there is no clear relationship between the levels of these hormones and stress-associated mental illnesses such as posttraumatic stress disorder (PTSD). Therefore, other hormones are likely to be involved in this effect of stress. Here we used a rodent model of PTSD in which rats repeatedly exposed to a stressor display heightened fear learning following auditory Pavlovian fear conditioning. Our results show that stress-related increases in circulating ghrelin, a peptide hormone, are necessary and sufficient for stress-associated vulnerability to exacerbated fear learning and these actions of ghrelin occur in the amygdala. Importantly, these actions are also independent of the classic HPA stress axis. Repeated systemic administration of a ghrelin receptor agonist enhanced fear memory but did not increase either corticotropin-releasing factor (CRF) or corticosterone. Repeated intraamygdala infusion of a ghrelin receptor agonist produced a similar enhancement of fear memory. Ghrelin receptor antagonism during repeated stress abolished stress-related enhancement of fear memory without blunting stress-induced corticosterone release. We also examined links between ghrelin and growth hormone (GH), a major downstream effector of the ghrelin receptor. GH protein was upregulated in the amygdala following chronic stress, and its release from amygdala neurons was enhanced by ghrelin receptor stimulation. Virus-mediated overexpression of GH in the amygdala was also sufficient to increase fear. Finally, virus-mediated overexpression of a GH receptor antagonist was sufficient to block the fear-enhancing effects of repeated ghrelin receptor stimulation. Thus, ghrelin requires GH in the amygdala to exert fear-enhancing effects. These results suggest that ghrelin mediates a novel branch of the stress response and highlight a previously unrecognized role for ghrelin and growth hormone in maladaptive changes following prolonged stress.

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INTRODUCTION

When a cue or event threatens the well-being of an organism, stress responses are engaged to promote coping and adaptation.[1] Despite the utility of these responses, repeated or prolonged activation of the stress response causes detrimental effects, including increased susceptibility to mental illnesses such as depression, anxiety or posttraumatic stress disorder (PTSD).[2–8] It is thought that the stress response is principally coordinated by hormones of the hypothalamic–pituitary–adrenal (HPA) axis; however, strong correlations between stress-induced alterations in these hormones and mental illness are lacking, and not all effects of chronic stress can be simulated with exogenous administration of the HPA hormones.[9,10] Furthermore, although excessive HPA activity has been linked to heightened fear and anxiety in rodents, there has been little success in the clinical application of these findings.[11] Both high and low levels of HPA activity have been observed in humans with stress-sensitive mental disorders and, in some cases, patients respond positively to treatment with exogenous glucocorticoids, one of the adrenal stress hormones.[12] Thus, there is a crucial need for novel biomarkers and therapeutic targets.

Recently, it has been found that ghrelin, a peptide hormone, is modulated by exposure to stress.[13,14] Ghrelin is produced by the stomach where it is activated by posttranslational acylation before being transported into the blood stream. It can then cross the blood–brain barrier[13] where it binds to the growth hormone secretagogue receptor 1a (GHSR1a, or ghrelin receptor). Interestingly, the ghrelin receptor is found in the basolateral complex of the amygdala (BLA),[15] a brain region that regulates negative emotional states such as fear. Single infusions of exogenous ghrelin into the amygdala can alter behavior in tasks such as the elevated plus maze and inhibitory avoidance,[16] but a relationship between emotional learning and endogenous ghrelin has not been explored. Furthermore, growth hormone (GH), which is released by cells in response to ghrelin receptor activation and altered in the brain by stress,[17] is present in BLA neurons.[18] The stress sensitivity of ghrelin, together with the localization of its receptor and downstream signaling partner (GH) in BLA, make it an attractive candidate mechanism by which emotional memories may be altered following periods of stress.

PTSD and other trauma- and stress-related disorders can arise following a traumatic experience.[19] Chronically stressed individuals are especially vulnerable to developing these disorders in response to trauma.[3,8,20,21] Humans with PTSD and other anxiety disorders exhibit hyperactivity in the amygdala,[22,23] and amygdala-dependent processes, such as fear learning to novel stimuli in laboratory settings, are enhanced in humans with

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PTSD.[24] The 'over-acquisition' of aversive memories is not merely a symptom of trauma-related disorders. It is also thought to contribute to the genesis of such disorders and may perpetuate distress after the trauma: humans with PTSD have extremely strong memories of the PTSD-inducing trauma, and many symptoms of PTSD, such as social avoidance and sleep disturbance, are secondary to these memories.[25–27] Thus, understanding why certain individuals, such as those with a significant lifetime stress burden, have dysregulated encoding of traumatic memories is critical for both preventing and treating PTSD and other trauma-related disorders. In addition, by understanding how stress alters brain regions responsible for emotional memory such as the amygdala, we may better understand how stress increases susceptibility to the development of other psychiatric disorders.

In this paper, we used a rodent model of PTSD in which rats were repeatedly exposed to stress and then subjected to auditory Pavlovian fear conditioning. Relative to unstressed controls, these animals displayed enhanced fear learning, mimicking the stress-induced vulnerability to excessive learning about aversive experiences that is a key feature in the acquisition and symptomatology of PTSD. Our studies uncover an essential and novel role for ghrelin and growth hormone in stress-induced susceptibility to exacerbated fear.

MATERIALS AND METHODS

Subjects

All experiments used adult male Long Evans rats (250–350 g, Taconic, Germantown, NY, USA), housed individually (68–72 °F; 12-h light/dark cycle, 0700 h lights on). Food and water (or 0.9% saline for adrenalectomy (ADX) experiments) was provided *ad libitum*. Stressed and unstressed animals were housed in separate cubicles. All procedures were in accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Massachusetts Institute of Technology and the Animal Care and Use Review Office of the US Army Medical Research and Materiel Command.

Surgical procedures

Some rats received ADX, cannulae implants or virus infusions, as described in Supplementary Information Methods.

Virus preparation

Herpes simplex virus-based amplicons for the overexpression of growth hormone were previously constructed and characterized.[28] Virus was packaged with the 5*dl*1.2 helper virus and 2-2 cells using standard methods as described in Supplementary Information Methods.[29] Virus was purified on a sucrose gradient, pelleted and resuspended in 10% sucrose in Dulbecco's phosphate-buffered saline. Titers were $\sim 1 \times 10^8$ IU ml^{-1}.

Adeno-associated viral plasmids were constructed as described in Supplementary Information Methods and sent to Virovek (Hayward, CA, USA) for packaging with AAV1/2 chimeric pseudotyping. Purified viruses were suspended in 10% sucrose in Dulbecco's phosphate-buffered saline and titers were 2.4×10^{12} vg ml^{-1}.

Drug preparation

For systemic drug delivery, rats were injected with 1 ml kg^{-1} (intraperitoneal) of the appropriate solution. All drugs were solubilized in 0.9% saline (vehicle) such that injection volumes remained constant for each experiment. MK-0677 (also known as ibutamoren mesylate; Merck, Whitehouse, NJ, USA, or Axon Medchem, Groningen, The Netherlands) is a highly specific GHSR1a agonist that readily crosses the blood–brain barrier and has a half-life of >6 h.[30,31] A concentration of 0.5 mg ml^{-1}, diluted in vehicle, was selected because it is well tolerated and results in significant and prolonged increases in growth hormone release.[31] D-Lys^3-GHRP-6 (DLys3, Tocris Biosciences, Minneapolis, MN, USA) was diluted to 2.74 μg ml^{-1} in vehicle. DLys3 is a selective and potent inhibitor of GHSR1a[32,33] with the half-maximal inhibitory concentration of 0.9 μM[34] (Tocris Bioscience literature). It also crosses the blood–brain barrier.[35] The only other known receptor class with affinity for DLys3 are the melanocortin receptors, but $K_i = 26$–120 μM, and hence the dilute dose used here would not be expected to affect these receptors. DLys3 was injected within 30 min of the start of immobilization stress or following handling.

For experiments using intra-BLA drug delivery, drugs were solubilized in physiological artificial cerebrospinal fluid (vehicle; pH = 7.35). MK-0677 was solubilized to 0.5 μg μl^{-1}. For bioactive ghrelin, a dose of 5 nmol μl^{-1}, diluted in vehicle, was selected as it was previously shown to have behavioral effects following a single infusion into the amygdala.[16]

Drug infusion

For intracranial infusions, rats were placed in 18.9 l buckets containing bedding. The dummy cannulae were removed and injectors (30G stainless steel cannulae; extending 1 mm beyond the cannulae end) were inserted. The injectors were attached to Hamilton syringes (10 μl; Hamilton, Reno, NV, USA) via polyethylene tubing, and the syringes were mounted in a Harvard syringe pump (Harvard Apparatus, Holliston, MA, USA). Infusions were given at a rate of 0.1 μl m^{-1} for 5 min for a total volume of 0.5 μl per side, with 1 min for diffusion, before the injectors were removed and new dummy cannulae were inserted.

Stress exposure

Some rats were exposed to either immobilization stress or water stress, as described in Supplementary Information Methods.

Behavioral testing

Some rats were subjected to either auditory Pavlovian fear conditioning or the elevated plus maze, as described in Supplementary Information Methods.

Histology

Following completion of the experiment, animals were anesthetized with an overdose of isoflurane and intracardially perfused with physiological saline followed by 4% formalin fixative in saline. Perimortem blood was collected from some rats, as described in Supplementary Information Methods. Brains were harvested and placed in 4% formalin for 24–72 h. The brains were then transferred to a 30% sucrose/4% formalin solution for a minimum of 3 days. For brains infused with virus, solutions containing paraformaldehyde were used in lieu of formalin. Coronal sections (40 μm) were made and mounted on gelatinized slides. Tissue that did not contain virus was stained with 0.1% cresyl violet. Slides were then assessed for cannulae position or green fluorescent protein (GFP) fluorescence. Animals with incorrect placements were excluded from all analyses.

Hormone assays

Corticosterone, acylated ghrelin, growth hormone and corticotrophin-releasing factor (CRF) levels were determined using commercial ELISA kits, as described in Supplementary Information Methods.

Statistics

For each fear memory session, conditional freezing was assessed as a percentage of time spent freezing, a probability estimate that is amenable to analysis with parametric statistics. These probability estimates of freezing, along with other measures, were analyzed using analysis of variance. The *post hoc* comparisons in the form of Fisher's protected least significant difference tests were performed after a significant omnibus F-ratio ($P < 0.05$). Statistical trends are noted in the text when omnibus F-ratio did not reach $P < 0.05$ but were $P < 0.10$. All data where $P > 0.10$ were identified as not significant (*ns*).

RESULTS

Stress-related changes in fear and ghrelin are independent of adrenal stress hormones

We used an animal model of PTSD in which rats were repeatedly exposed to immobilization stress (4 h per day for 14 days) and subsequently administered auditory fear conditioning. Many studies suggest that the development of affective illness following stress, including disorders involving fear,[9,10,36] is because of repeated activation of the HPA axis, which results in elevated

adrenal stress hormone release.[6,37] To determine whether stress-induced increases in fear learning require adrenal stress hormones, such as corticosterone or adrenaline, we examined the impact of ADX on stress-related enhancement of fear conditioning. Following ADX or sham surgery (SHAM), animals were exposed to immobilization stress (STR) or daily handling (no stress (NS)). One subset of animals underwent auditory fear conditioning 24 h after the final stress or handling session. Fear to the tone was assessed 48 h after conditioning. Although a slight enhancement of fear acquisition was seen in stressed rats, this did not reach statistical significance (Figure 1a, stress: $F(1, 22) = 3.98$, $P < 0.10$). However, stress produced a robust enhancement of long-term fear memory when fear to the tone was later tested in a novel context (Figure 1b, stress: $F(1, 22) = 12.17$, $P < 0.01$). Surprisingly, the fear-enhancing effect of stress was observed in the complete absence of adrenal stress hormones (Figure 1b, surgery × stress interaction, $F(1, 22) = 1.3$, $P = ns$; corticosterone verified as undetectable in all ADX animals, Figure 1c).

The enhancement in long-term fear memory after stress cannot be explained by stress-related changes in extinction nor memory retrieval: no difference in extinction retention was observed in a second extinction test performed 48 h after the initial extinction session (Supplementary Figure 1a; stress: $F(1, 12) = 2.15$, $P = ns$), and stress administered after fear conditioning did not alter the expression of previously acquired fear memories (Supplementary Figure 1b; stress: $F(1, 17) = 0.107$, $P = ns$). The high levels of conditional freezing seen in rats in the STR or ADX groups also cannot be explained by nonspecific decreases in locomotor activity (Supplementary Figure 2a; stress: $F(1, 22) = 1.52$, $P = ns$ and surgery: $F(1, 22) = 0.56$, $P = ns$) or increases in spontaneous freezing (Supplementary Figure 2b; stress: $F(1, 22) = 0.37$, $P = ns$ and surgery: $F(1, 22) = 0.23$, $P = ns$).

Stress-related enhancement of fear required multiple sessions and was not due to the most recent stress session: a single session of immobilization stress was not sufficient to increase subsequent fear learning (Supplementary Figure 3a). Stress-related enhancement of fear also did not stem from delayed effects of the first stress exposure as has been shown in other aspects of stress:[9,38] a single exposure to immobilization stress did not affect fear conditioning administered 14 days later (Supplementary Figure 3b, stress: $F(1, 6) = 2.90$, $P = ns$). Rather, stress-related increases in fear memory appeared after cumulative stress exposure of a approximately five or more days for this particular stressor (Supplementary Figure 3a and c, group: $F(5, 46) = 5.01$, $P < 0.01$ and $F(1, 13) = 4.62$, $P < 0.10$), respectively). These results are consistent with the findings that stress 'load' and neuronal dysfunction are correlated.[39]

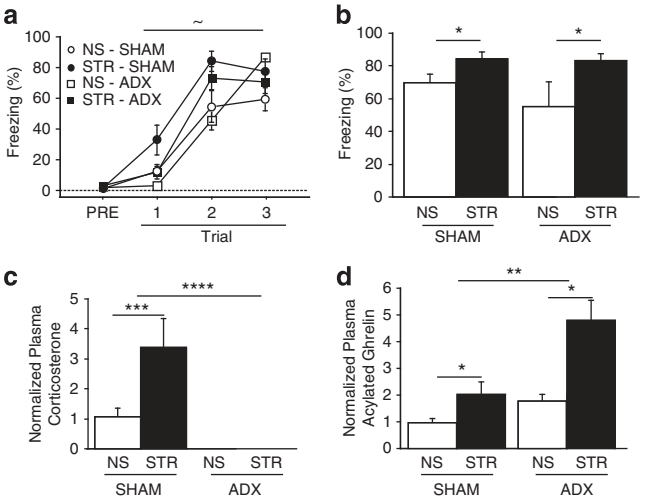

Figure 1. Stress-related changes in fear and ghrelin are independent of adrenal stress hormones. Animals received adrenalectomy (ADX) or sham surgery (SHAM). After at least a week of recovery, animals received either 14 days (4 h per day) of immobilization stress (STR) or gentle handling (no stress (NS)). (**a**) Some animals received auditory Pavlovian fear conditioning 24 h after the last stress or handling session. (**b**) Fear to the tone was assessed 48 h later in a novel context. In a separate group of animals, trunk blood was collected 24 h after the last stress session. Plasma level corticosterone (**c**) and acylated ghrelin (**d**) were determined with enzyme-linked immunosorbent assay (ELISA). All data are mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $^{\sim}P < 0.10$ in planned comparisons.

Because hormones of the HPA axis coordinate so many aspects of the stress response, it is surprising that stress-enhanced fear learning is not mediated by glucocorticoids or adrenaline. Interestingly, this is consistent with the limited clinical benefit of pharmacological manipulations targeting adrenal hormone signaling in PTSD patients.[12,40] Importantly, these data also suggest that other stress hormones drive stress-related changes in aversive learning and memory.

Circulating acylated ghrelin is elevated following chronic stress,[13] raising the possibility that it may function as a stress hormone but its relationship with HPA hormones is poorly defined. To clarify this, we examined the impact of ADX on stress-induced increases in acylated ghrelin. Animals were administered surgical and stress treatments as per the previous experiment but killed 24 h after the final stress or handling session for the collection of blood samples. This was performed during a narrow window surrounding the circadian trough of ghrelin release to minimize hunger-induced variability in ghrelin levels. Corticosterone was significantly elevated by immobilization stress in the SHAM group but undetectable in the ADX group, as expected (Figure 1c, stress × surgery interaction: $F(1, 17) = 8.37$, $P < 0.05$). In contrast, acylated ghrelin was elevated by stress regardless of the presence or absence of the adrenal glands (Figure 1d, stress: $F(1, 17) = 13.19$, $P < 0.01$, and stress × surgery interaction: $F(1, 17) = 2.99$, $P = ns$). Interestingly, stress-related increases in acylated ghrelin were amplified by ADX (Figure 1d, surgery: $F(1, 17) = 9.97$, $P < 0.01$), showing that adrenal hormones actually inhibit, rather than facilitate, ghrelin release.[41] In addition, the elevation of ghrelin by stress in the absence of adrenal hormones raises the intriguing possibility that the ghrelin pathway mediates stress-related enhancement of fear.

Ghrelin is elevated not only by psychological stressors such as immobilization stress, but also by other stressors, including those induced by environmental factors (water stress, Supplementary Figure 4, stress: $F(1, 14) = 33.46$, $P < 0.0001$) and social status (social defeat[13]). Also, just as a single immobilization session does not lead to enhanced fear learning 24 h later, a single immobilization session also does not elevated circulating ghrelin (Supplementary Figure 5, stress: $F(1, 11) = 0.01$, $P = ns$). Together, our data reveal that ghrelin is not simply a downstream effector of adrenal hormone recruitment during chronic stress, but may instead represent an independent hormonal pathway of the stress response, broadly recruited by different stressors.

Repeated activation of the ghrelin receptor is sufficient for enhanced fear in the absence of stress and independent of the HPA axis

To determine whether increased activation of the ghrelin receptor is sufficient for enhancement of fear memory, we conducted experiments using pharmacological agonism of GHSR1a in nonstressed animals. Stress-induced changes in acylated ghrelin were observed at the nadir of the diurnal ghrelin cycle, suggesting that stress-related increases in ghrelin persist throughout the day. Because the half-life of acylated ghrelin is short (~30 min[42]), we used MK-0677 (also known as ibutamoren mesylate), a highly

selective GHSR1a agonist with a half-life of at least 5–6 h,[43] instead of exogenous acylated ghrelin in order to more closely model the prolonged stress-induced increases in GHSR activation by endogenous ghrelin. We systemically administered MK-0677 (MK: 5 days) or saline (VEH: 5 days) once a day for five consecutive days in nonstressed rats to determine whether repeated ghrelin receptor agonism in the absence of stress is sufficient to increase fear learning and whether HPA hormones may play a role in this effect. Five days of treatment were used because this reflects the minimum number of sessions our immobilization stress must be repeated to see stress-related enhancement of fear (Supplementary Figure 3a and c). A subset of animals was administered auditory fear conditioning 24 h after the last injection. This drug regimen did not alter acquisition during conditioning (Figure 2a, injection: $F(1,31)=1.54$, $P=ns$), but did significantly enhance long-term fear memory (Figure 2b, injection: $F(1,31)=4.21$, $P<0.05$). This enhancement was similar to the effect of chronic immobilization stress and was not attributable to a drug-induced increase in spontaneous freezing (Supplementary Figure 6a, injection: $F(1,31)=0.25$, $P=ns$) or a drug-induced decrease in locomotor activity (Supplementary Figure 6b and c, injection: $F(1,31)=0.95$, $F(1,15)=2.44$, $P=ns$ all). In addition, it was specific to associative aversive processing, as innate anxiety was not altered (Supplementary Figure 6d, treatment; $F(1,15)=0.15$, $P=ns$).

Furthermore, just as we observed following chronic immobilization stress (Supplementary Figure 1b), fear expression was not altered following chronic ghrelin receptor agonism: previously acquired auditory fear memory was not affected by chronic ghrelin receptor agonism (Supplementary Figure 6e, injection: $F(1,10)=0.30$, $P=ns$). In addition, the enhancement of fear memory by repeated ghrelin receptor agonism cannot be attributed to effects of the most recent drug treatment (Supplementary Figure 7a, injection; $F(1,19)=3.70$, $P<0.10$) or delayed effects arising from the first drug treatment (Supplementary Figure 7b; injection: $F(1,6)=0.22$, $P=ns$). Interestingly, there is a trend toward impairment, and not enhancement, of fear learning after a single dose of the ghrelin receptor agonist (Supplementary Figure 7a). This effect is similar to the effect of a single immobilization session (see Supplementary Figure 3a) and is also

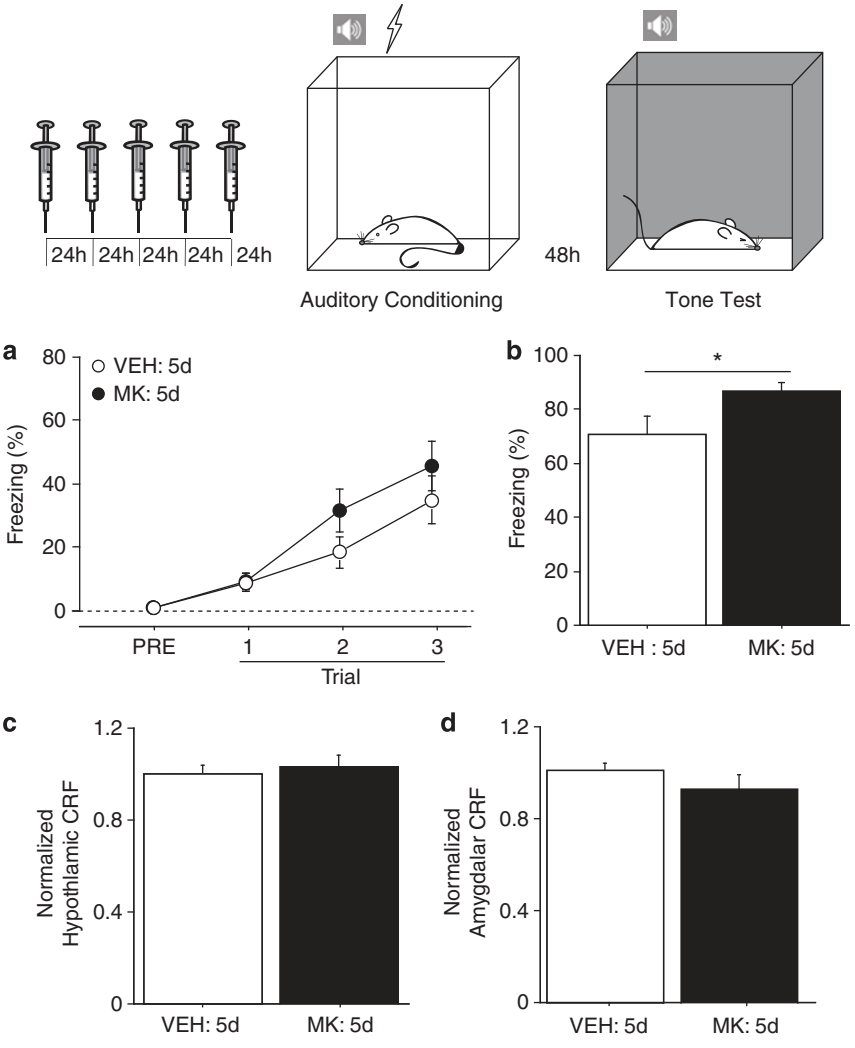

Figure 2. Long-term pharmacological stimulation of ghrelin receptor activity enhances fear memory without altering other stress hormones. Rats received daily systemic injections of MK-0677 (MK: 5 days (5d)), a GHSR1a agonist, or saline (VEH: 5 days) for 5 days at the endogenous ghrelin signaling nadir. (**a**) One group underwent auditory fear conditioning 24 h following the final injection. Fear acquisition was assessed by monitoring freezing levels. (**b**) Conditional freezing to the tone was assessed in a novel context 48 h following fear conditioning. A separate group was killed 24 h following the final injection and microdissections of hypothalamus and amygdala were performed. Brain corticotropin-releasing factor (CRF) levels were measured using enzyme-linked immunosorbent assay (ELISA). (**c**) Hypothalamus and (**d**) basolateral complex of the amygdala. $^*P<0.05$ in planned comparisons.

consistent with other reports that ghrelin may reduce aversive processing following acute stress.[44] These data suggest that long-term activation of the ghrelin receptor is sufficient to enhance fear memory, whereas short-term activation of the ghrelin receptor may actually impair fear memory.

Although stress-related increases in ghrelin are not triggered by the HPA axis, ghrelin could interact with the HPA axis in other ways to enhance fear. For example, the hypothalamic stress hormone CRF is secreted by neurons of the paraventricular nucleus, an area dense with GHSR1a.[45] Moreover, hypothalamic CRF neurons project to the amygdala and amygdalar CRF can modulate fear memory.[46,47] Thus, systemic ghrelin receptor agonism could mediate effects on fear learning by increasing CRF release in the amygdala. In addition, ghrelin receptors have been identified in the adrenal cortex.[48] Therefore, systemic ghrelin receptor agonism could mediate effects on fear learning by increasing release of adrenal hormones. To determine whether the effects of ghrelin on fear learning are mediated through the HPA axis, we examined CRF peptide levels in both the hypothalamus and the amygdala of animals treated as above. There was no change in hypothalamic CRF (Figure 2c, injection: $F(1, 21) = 0.20$, $P = ns$) or amygdalar CRF (Figure 2d, injection: $F(1, 21) = 1.3$, $P = ns$). In a third group of animals, we examined adrenal weights following a more prolonged period of ghrelin receptor agonism. Increased adrenal weight is seen following prolonged recruitment of adrenocorticotrophin from the pituitary because it triggers enhanced glucocorticoid and adrenaline production and release from the adrenal glands. Animals received systemic administration of MK-0677 (MK: 14 days) or saline (VEH: 14 days) once a day for 14 days. Repeated systemic ghrelin receptor agonism did not alter adrenal weight (Supplementary Figure 8, injection: $F(1, 14) = 1.24$, $P = ns$). This suggests that repeated ghrelin receptor agonism at the dose used here does not stimulate the HPA axis.

Fear memory requires plasticity in numerous brain regions but the BLA is particularly important for both formation and storage of learned fear. Recently, it has also been identified as the region of the amygdala with the highest density of ghrelin receptors.[15] To determine whether repeated ghrelin receptor activation in the BLA is sufficient to enhance fear memory, we infused either MK-0677 (MK-Inf: 5 days) or artificial cerebrospinal fluid (vehicle, VEH-Inf: 5 days) directly into the BLA daily for 5 days before auditory fear conditioning. Freezing during fear conditioning was not altered by the treatment (Figure 3a, infusion: $F(1, 8) = 0.36$, $P = ns$) but long-term fear memory was significantly enhanced (Figure 3b, infusion: $F(1, 8) = 13.75$, $P < 0.01$). A similar potentiation of fear memory was observed when acylated ghrelin was infused into the BLA daily for 5 days (Supplementary Figure 9; infusion: $F(1, 8) = 6.07$, $P < 0.05$). Collectively, these data show that repeated activation of the ghrelin receptor directly in BLA is sufficient for heightened fear memory. The ability of the repeated intraamygdala infusions to fully recapitulate the effects of repeated systemic ghrelin agonist delivery strongly suggests that stress-induced increases in circulating ghrelin enhance fear through direct actions in the BLA. In addition, because direct intra-BLA manipulations are unlikely to increase either CRF or adrenocorticotrophin,[49] this provides further support for the claim that ghrelin alters fear by direct actions in the amygdala, rather than through interactions with the HPA axis or ghrelin receptors in the periphery.

The ghrelin pathway is necessary for stress-induced vulnerability to fear during chronic stress

To determine whether ghrelin signaling is necessary for stress-related enhancement of fear memory, we blocked ghrelin receptor signaling during repeated stress sessions. Rats were administered immobilization stress (STR) or daily handling (NS) and given either a systemic injection of DLys3, a highly specific inverse agonist of GHSR-1a that crosses the blood–brain barrier, or saline (VEH) at the start of each session.[43] At 24 h following the final stress or handling session, we administered auditory fear conditioning and assessed fear to the tone in a subsequent

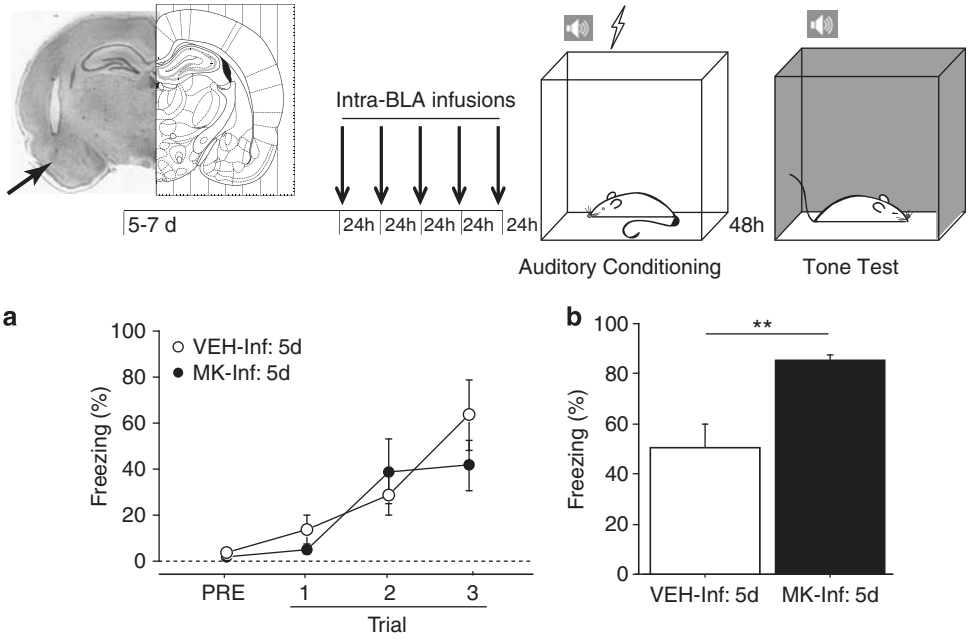

Figure 3. Long-term pharmacological stimulation of ghrelin receptor activity in the amygdala enhances fear memory. Rats were implanted with bilateral cannulae aimed at the basolateral complex of the amygdala (BLA). The arrow indicates the tip of the injector within a representative coronal brain section. Following recovery, intra-BLA infusions of either MK-0677 (MK-Inf: 5 days (5d)) or artificial cerebrospinal fluid (VEH-Inf: 5d) were administered daily for five consecutive days and, 24 h following the final infusion, (**a**) auditory fear conditioning was administered. (**b**) Fear memory was assessed in a novel context 48 h following fear conditioning. Brain illustration is adapted from Paxinos and Watson.[88] All data are mean ± s.e.m. $^{**}P < 0.01$ in planned comparisons.

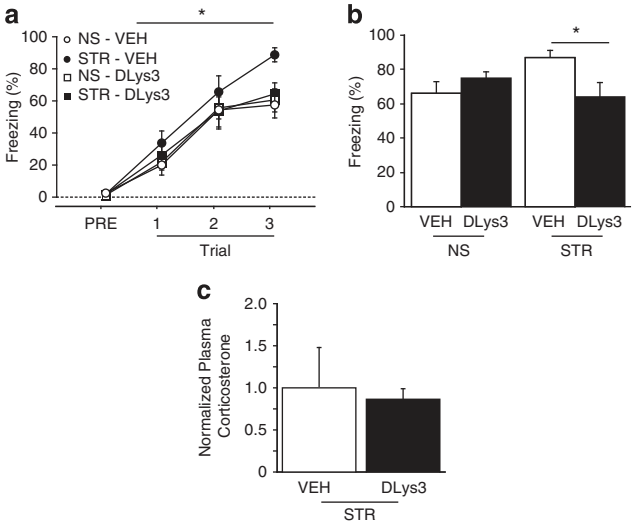

Figure 4. Ghrelin receptor antagonism during chronic stress abolishes stress-related enhancement of fear memory without affecting corticosterone release. Rats received either daily handling (no stress (NS)) or immobilization stress (STR). Each day, a systemic injection of either D-Lys^3-GHRP-6 (DLys3), an antagonist of GHSR1a, or saline (VEH) was administered within 30 min of handling or stress initiation. (**a**) Animals received auditory fear conditioning 24 h after the last stress or handling session. (**b**) Fear memory was assessed 48 h after the conditioning session by placing the animals in a novel context and measuring conditional freezing during tone presentation. (**c**) In a subset of animals in the STR group, tail bleeds were performed during the final 30 min of the final stress session and plasma corticosterone levels were measured using enzyme-linked immunosorbent assay (ELISA). All data are mean ± s.e.m. $^*P<0.05$ in planned comparisons.

session. Stress enhanced fear acquisition in the saline-treated group (Figure 4a; for trial 3, stress: $F(1,27)=6.75$, $P<0.05$, and planned comparisons, STR-VEH vs NS-VEH, $P<0.05$) but this effect was not seen when the ghrelin receptor was antagonized during immobilization stress (Figure 4a, trial 3, stress × injection interaction: $F(1,27)=3.94$, $P<0.10$ and planned comparisons, STR-DLys3 vs STR-VEH). Long-term fear memory was enhanced by stress in saline-treated control animals as seen previously (Figure 1b and Supplementary Figure 3a), demonstrating that any stress from injections did not alter this effect,[50] but DLys3 completely reversed stress-enhanced fear (Figure 4b; injection × stress interaction: $F(1,27)=6.36$, $P<0.05$ and planned comparisons, STR- DLys3 vs STR-VEH). In contrast, ghrelin receptor antagonism had no effect on fear memory in nonstressed controls (Figure 4b; planned comparison, NS-SAL vs NS-DLys3). Moreover, DLys3 treatment did not blunt stress-induced HPA activation, demonstrated by similar levels of stress-induced corticosterone between DLys3- and vehicle-injected rats (Figure 4c; injection: $F(1,5)=0.10$, $P=ns$). These data show that ghrelin-mediated signaling is necessary for stress-related enhancement of fear and suggest, surprisingly, that other peripheral or central stress hormones are not sufficient to mediate this effect in the absence of heightened ghrelin signaling.

Growth hormone, a major effector of the ghrelin receptor, interacts with ghrelin in the amygdala to enhance fear memory

One of the best-characterized consequences of ghrelin receptor activation is release of GH.[51] Although the pituitary expresses the highest levels of GH, it is also expressed in other brain regions, including the BLA.[18] In one limbic region, the hippocampus, GH levels have been shown to increase following acute stress.[17] However, it is not known how prolonged stress alters GH in the BLA. To test this, we examined the impact of repeated immobilization stress (STR) or daily handling (NS) on GH levels in the BLA. We found that GH was readily detected in BLA homogenate and significantly upregulated 24 h after chronic stress (Figure 5a, group: $F(1,16)=6.44$, $P<0.05$), the time point at which we observe increases in circulating ghrelin and fear conditioning. This suggests that GH-mediated signaling in the BLA may be amplified following stress.

GH can induce synaptic plasticity[52] and is increased in response to learning,[53] but it is unclear how it affects amygdala function. Herpes simplex-based viral vectors were used to express recombinant rat GH and a GFP reporter or GFP only.[28] Naive rats received intra-BLA infusions of either the recombinant rat GH virus or the GFP-only control virus (CON) (Figure 5b). After 3 days, when herpes simplex virus-mediated transgene expression is at its maximum,[54] auditory fear conditioning was administered. Fear to the tone was assessed 48 h later. Overexpression of recombinant rat GH did not alter fear acquisition (Figure 5c; infusion × trial interaction: $F(4,52)=0.57$, $P=ns$) but did enhance long-term fear memory (Figure 5d, infusion: $F(1,13)=9.97$, $P<0.01$). These data demonstrate that high levels of GH in the BLA are sufficient to enhance fear learning, an effect that is similar to the effect of repeated intra-BLA ghrelin receptor stimulation (Figure 3 and Supplementary Figure 9).

To determine whether ghrelin receptor stimulation in amygdala triggers the release of GH as it does from the pituitary,[51] we generated short-term cultures of BLA cells and measured GH protein in the media following treatment with either MK-0677 or vehicle. Stimulation of the ghrelin receptor in BLA cells led to significantly elevated release of GH (Figure 5e; treatment: $F(1,8)=8.24$, $P<0.05$). These results show that ghrelin receptor stimulation can trigger GH release from the amygdala.

Because ghrelin and GH can interact in amygdala, we next sought to determine whether GH is a necessary downstream signaling partner for the fear-enhancing effects of repeated ghrelin receptor stimulation. We generated an AAV viral construct to overexpress a mutant form of the rat GH protein (GHA) that acts as a functional antagonist to endogenous GH.[55,56] Following infusion of AAV to overexpress either GHA or a control protein (GFP) (Figure 5f), rats were permitted to recover for 5 weeks. After recovery, rats that were infused with GHA received daily injections of either the ghrelin receptor agonist MK-0677 (MK) or saline (SAL) for 10 days. Rats that were infused with GFP received daily injections of SAL for 10 days. At 24 h after the final injection, all rats were subjected to auditory fear conditioning, followed by assessment of long-term auditory fear memory in a subsequent session 48 h later. Although no differences were observed between any group during fear conditioning (Figure 5g; group: $F(2,13)=0.07$; $P=ns$), differences in long-term fear memory were observed (Figure 5h; group: $F(2,13)=4.32$, $P<0.05$). Specifically, antagonizing the activity of GH prevented the fear-enhancing effects of repeated ghrelin receptor agonism (Figure 5h; planned comparisons between GFP-SAL vs GFP-MK and GFP-MK vs GHA-MK). These data reveal that repeated ghrelin receptor stimulation requires GH-dependent signaling in the amygdala to exert its fear-enhancing effects.

DISCUSSION

In conclusion, we first show that ghrelin acts in parallel to the HPA axis: ADX does not affect the ability of stress to enhance fear learning or increase circulating acylated ghrelin. This finding indicates that these effects of stress are not simply downstream from glucocorticoids or adrenal catecholamines. We also show that increased ghrelin receptor activity is sufficient and necessary for stress-enhanced fear and is dissociable from HPA activity. Repeated activation of ghrelin receptors in nonstressed animals significantly enhances fear learning without elevating HPA stress

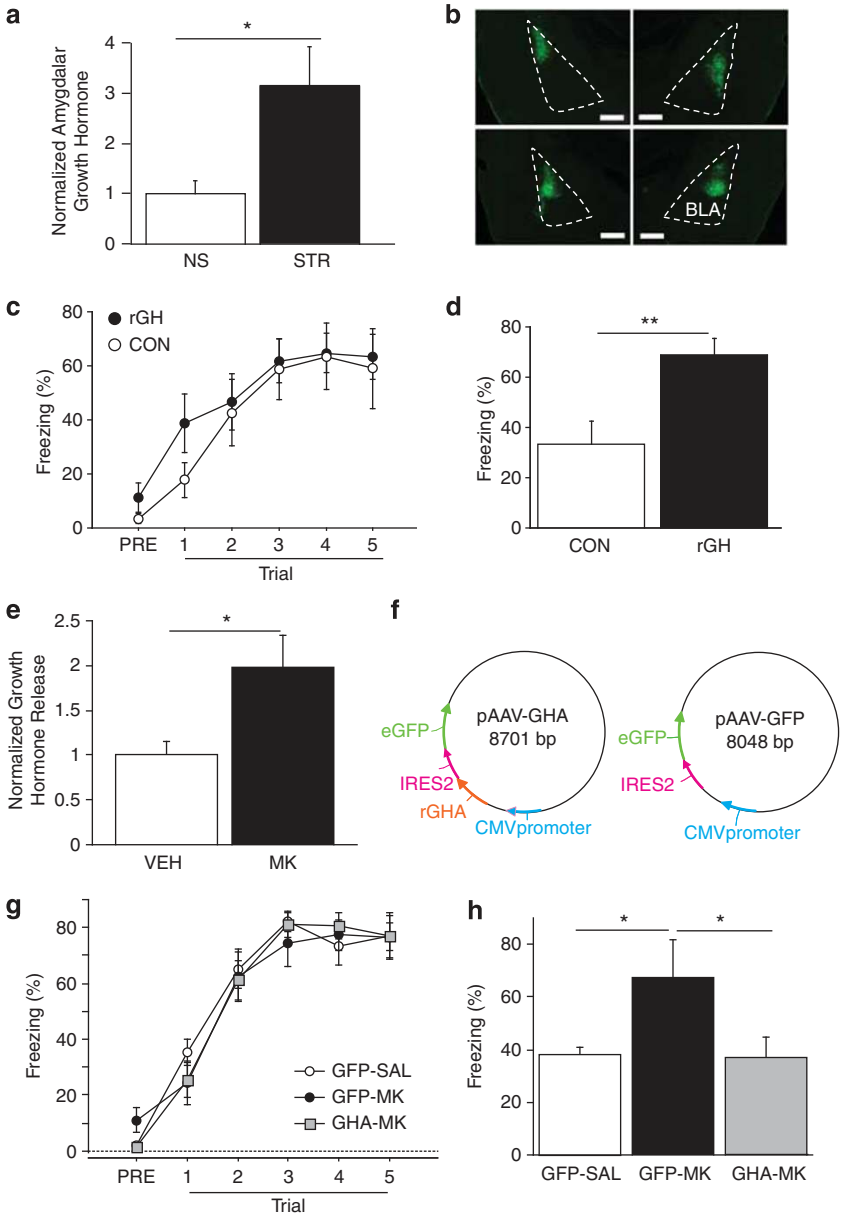

Figure 5. Amygdalar growth hormone is increased by chronic stress, is sufficient to enhance fear memory and is necessary for the fear-potentiating effects of ghrelin receptor stimulation. (**a**) Rats received either daily handling (no stress (NS)) or immobilization stress (STR) for 14 days. Animals were killed 24 h after the last stress or handling session and the basolateral complex of the amygdala (BLA) was dissected. Growth hormone (GH) levels in the BLA were measured using enzyme-linked immunosorbent assay (ELISA). (**b**) The herpes simplex virus (HSV)-based viral vectors[28] expressing either green fluorescent protein (GFP; CON) or recombinant rat GH (rGH) was infused in the BLA and expression was assessed after behavioral testing was complete. Representative GFP expression from two rats is shown. (**c**) Auditory Pavlovian fear conditioning was performed 3 days after HSV virus infusions. (**d**) Long-term fear memory was assessed by placing the animals in a novel context and measuring conditional freezing following tone presentation 48 h after the conditioning session. (**e**) Short-term BLA cell cultures were used to measure GH release following treatment with a ghrelin receptor agonist (MK) or vehicle (VEH). (**f**) Adeno-associated virus (AAV) constructs were generated to examine the contribution of GH-mediated signaling in the BLA to ghrelin-induced potentiation of fear. (**g**) Following infusion of the AAVs into the BLA and recovery, rats received 10 days of systemic injection of either a ghrelin receptor agonist (MK) or vehicle (VEH). After 24 h, auditory Pavlovian fear conditioning was administered to all rats. (**h**) Long-term fear memory was assessed by placing the animals in a novel context and measuring conditional freezing following tone presentation 48 h after the conditioning session. Scale bar, is 500 μm. All data are mean ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$ in planned comparisons.

hormones, whereas systemic blockade of the ghrelin receptor during chronic stress prevents stress-related enhancement of fear, even in the presence of elevated adrenal stress hormones. We demonstrate that the amygdala, a brain region that displays enhanced function in chronically stressed animals and in patients with trauma-related disorders, is likely the locus of the fear-enhancing effects of repeated ghrelin receptor stimulation. Finally, we show that GH, a downstream effector of ghrelin receptor activation, is increased in the BLA by chronic stress, is sufficient to enhance fear learning and plays a necessary role in the fear-potentiating effects of ghrelin. Thus, ghrelin and growth hormone act together in the amygdala to enhance fear.

Our study is the first to explicitly examine the effects of protracted exposure to elevated ghrelin, as observed following chronic stress. We show that there are profound differences in the behavioral consequences of ghrelin exposure following different exposure durations, similar to the cumulative nature of stress. We also provide the first evidence to link prolonged exposure to elevated ghrelin with a specific, detrimental consequence of stress, enhanced fear memory, which typifies trauma-induced anxiety disorders such as PTSD. Because PTSD is a multifaceted disorder producing many symptoms, including those related to avoidance and hyperarousal, it will be interesting to determine whether chronically elevated ghrelin contributes to these sequelae of PTSD in addition to promoting changes in fear learning and memory.

Our study is also the first to show that GH is a critical downstream mediator of the effects of ghrelin in amygdala. Such a relationship between ghrelin and GH has not been described outside of the pituitary.[51] We also provide the first evidence to link elevated amygdala GH with chronic stress and enhanced fear memory. Taken together, our data reveal that the amygdala may be especially sensitive to ghrelin-mediated effects of stress because chronic stress amplifies both ghrelin and GH.

In contrast to our findings that link ghrelin to a pathological condition, prior studies have argued that ghrelin promotes adaptive changes during stress, including antidepressant effects[13] and reduction in anxiety.[44] However, these studies are problematic because they either focused exclusively on acute ghrelin manipulations, which we show can have profoundly different effects from repeated ghrelin manipulations, or used short- and long-term ghrelin manipulations interchangeably. In addition, the alterations in ghrelin levels were achieved through artificial states: heightened ghrelin levels were attained by extreme food deprivation or a single bolus injection of the short-lived peptide. The antidepressant effect of ghrelin requires extremely high levels of ghrelin, as found in food-restricted rodents after 10–15% weight loss.[13] We find that this level of food deprivation leads to increased exploratory motor activity (Supplementary Figure 10; $F(1, 13) = 7.51$, $P < 0.05$). A recent study has also reported similar motor effects following acute ghrelin manipulations.[57] These motor effects can be a significant confound for measures that require locomotor activity, such as social interaction or exploration. Thus, the ghrelin may alleviate the psychomotor effects of depression in a manner similar to amphetamine.[58] It is also important to note that the antidepressant effect of ghrelin reported following a single injection of exogenous ghrelin was only a mild improvement of a stress-related impairment in social interaction;[13] enhanced ghrelin signaling did not promote 'normal' function following stress. Indeed, our results reported here are consistent with limited human data showing that patients with treatment-resistant major depressive disorder have higher ghrelin levels than control patients.[59]

Here we demonstrate changes in endogenous ghrelin following stress and also use a low-dose, long-acting agonist to replicate the naturally occurring ghrelin state. We also provide clear evidence that acute and chronic ghrelin receptor manipulations have profoundly different effects. It is important to note that the changes in fear reported here occurred following small, but persistent, changes in ghrelin signaling, and all were in the absence of any locomotor effects. We suggest that the utility of ghrelin in the stress response may be similar to glucocorticoids: under 'normal' conditions, there is an optimal level of the hormone,[60] and too little[61,62] or too much hormonal signaling[16] can lead to dysfunction in neuronal circuits. Repeated activation of ghrelin and glucocorticoid pathways together contributes to stress-induced 'load' on the body. In this regard, heightened ghrelin signaling may have both advantageous and undesirable consequences, but these must be carefully considered with respect to the length and level of elevated ghrelin exposure.

It is important to acknowledge the limitations of our rodent model of PTSD. In our experiments, rats were repeatedly exposed to immobilization stress to generate a vulnerability to high levels of fear learning and memory following Pavlovian fear conditioning. The unstressed rats that received fear conditioning displayed normal, adaptive levels of fear, and did not express PTSD-like changes. It is not clear whether repeated exposure to stress leads to PTSD *per se* in the absence of a specific aversive experience such as fear conditioning. For example, when rats are administered a single stressor repeatedly, many aspects of the stress response show habituation over the course of stress, suggesting that later stress exposures are less 'stressful' and perhaps less likely to give rise to PTSD.[63] In addition, the *Diagnostic and Statistical Manual of Mental Disorders V* requires that a diagnosis of PTSD be tied to a single traumatic event,[19] and not a history of aversive experiences, such as a gradual accumulation of significant life stressors like low socioeconomic status or abuse. However, populations with greater life stress accumulation demonstrate higher risk of both the occurrence of a traumatic event and the development of PTSD after trauma.[7,64] In animal models, when a single stressor is repeated, small changes during delivery of the stress, such as shifts in the environmental context in which it occurs,[65] are sufficient to relieve habituation. Thus, it is possible that even when a single type of stress is repeated over days or weeks, prior exposure to that stress can lead to changes in neural circuits that facilitate the mnemonic encoding of subsequent experiences of the stress. Consistent with this, rats exposed to chronic stress show physiological changes that are consistent with PTSD, including enhanced corticosterone responses to novel stressors,[66] sleep fragmentation,[67] decreased hippocampal volume,[68] anhedonia[69] and enhanced amygdala excitability.[70] As biomarkers for PTSD become better defined, it will be interesting to determine whether repeated stress exposure alone is sufficient to produce changes associated with PTSD before additional trauma exposure occurs.

The source of ghrelin that is important for modulating fear is unclear. The majority of ghrelin is synthesized and released by specialized endocrine cells lining the stomach, with a lesser quantity released by the small intestine.[71] However, significantly smaller amounts have been detected in a variety of other tissues, including the pancreas, lungs and kidneys.[72] Thus peripheral tissues likely contribute to the circulating levels of acylated ghrelin that we report here. Ghrelin may also be synthesized by small populations of neurons in the hypothalamus,[73] the cerebral cortex and the brainstem,[74] where it may act as a paracrine hormone rather than being secreted into the blood stream. However, immunoreactive ghrelin-containing fibers have never been reported in amygdala. Thus, it seems that the most likely source of bioactive ghrelin affecting fear lies in the periphery, although a role for centrally derived ghrelin cannot be fully eliminated.

Our results strongly suggest that the fear-modulating actions of repeated ghrelin receptor activation occur via direct actions in the lateral or basolateral divisions of the amygdala (BLA, together). Some studies have found that intrahypothalamic application of ghrelin can enhance activity in the amygdala, suggesting an indirect mechanism by which ghrelin can affect amygdala output, but this increased activity is observed only in the central nucleus of the amygdala,[75] which is downstream of the BLA. We show here that repeated direct intra-BLA application of a ghrelin receptor agonist was sufficient to mimic the fear-potentiating effects of chronic stress. Although GHSR1a is expressed throughout multiple nuclei of the amygdala, it is densest in the lateral nucleus and is expressed by the majority of cells in this region.[15] It is not clear whether this expression is restricted only to excitatory pyramidal neurons, or whether it may also be found in inhibitory interneurons; this is an exciting area for future research that will facilitate our understanding of the mechanistic underpinnings of ghrelin-related fear enhancement.

It is notable that our data show that ghrelin receptor antagonism did not modulate fear memory in unstressed animals. From a clinical standpoint, this is important because it suggests that antighrelin therapies aimed at reducing the stress response may have minimal impact on 'normal', adaptive fear learning. Our finding contrasts with one study in mice in which genetic ablation of the ghrelin receptor, GHSR-1a, produced a mild impairment of contextual fear memory when tested 1 month following fear conditioning.[76] The reason for this discrepancy is unclear. It may be that genetic ablation of the ghrelin receptor represents an extreme level of functional 'antagonism' of ghrelin signaling, and we might have observed slight fear memory deficits in unstressed animals if we used a high dose of the ghrelin receptor antagonist. Alternatively, the knockout of the ghrelin receptor from birth may have led to compensatory changes through development that were not present in our study.

It is not clear why acute and repeated ghrelin receptor stimulation have opposite effects on fear learning. Although GHSR1a activation engages excitatory G_q-dependent molecular cascades, GHSR1 also exhibits an extremely high level of constitutive activity in the absence of bound ligand.[77] Accordingly, transient stimulation of GHSR1a leads to rapid desensitization and internalization of the receptor that is slow to recover.[78] Such a change is consistent with the decreased fear learning we observed 24 h after a single injection of ghrelin receptor agonist. It is also consistent with the observation that transient bath application of ghrelin to lateral amygdala slices leads to decreased excitatory neurotransmission.[15] The electrophysiological changes elicited by chronic ghrelin receptor stimulation in amygdala are completely unexplored, but our work suggests that the change must be opposite to that seen after acute ghrelin receptor stimulation. We suggest that the internalization of the ghrelin receptor may habituate[63] following either chronic administration of ghrelin receptor agonist or chronic stress exposure. The differences in receptor kinetics following acute versus chronic ghrelin receptor stimulation represent an especially promising area for future research.

It is also not clear how ghrelin receptor activation changes the amygdala to produce changes in fear learning. Repeated ghrelin receptor stimulation enhances neuronal spine density and facilitates long-term potentiation,[61] effects that are also observed following chronic stress.[79,80] GH has also been shown to enhance long-term potentiation,[52,81] yet the links between GH and spine density have never been reported. It will be interesting to determine whether ghrelin requires GH for its effects on synaptic plasticity, as it does for its effects on fear learning.

Our data show that stress-related changes in amygdala-based aversive processing are not dependent on HPA activity and that ghrelin plays an important role in stress-related affective dysfunction by actions independent of the HPA axis. It is important to acknowledge that we did not determine whether the fear-potentiating effect of stress is mediated via HPA-independent actions of ghrelin in adrenalectomized animals. For our purposes, the most important issue was to address the mechanisms by which chronic stress enhances fear under 'normal' physiological circumstances, rather than how stress enhances fear in animals lacking adrenal hormones. We do not discount the role of the HPA axis in coordinating other aspects of the stress response, nor the importance of adrenal hormones in regulating amygdala function in animals without a history of prolonged stress exposure. It is clear that the HPA hormonal cascade mediates many of the consequences of stress (for review, see Rodrigues *et al.*[82]) and that signaling through glucocorticoid and adrenergic receptors is important for regulating amygdala function (see Roozendaal and McGaugh[83] and Krugers *et al.*[84] for review). However, this work may need to be re-examined through the lens of putative parallel stress pathways such as ghrelin. Future work will be needed to explore the possible synergistic effects of coactivation of the HPA axis and the ghrelin system during chronic stress.

No current treatments exist for preventing stress-related affective disorders, suggesting that our most intriguing and important finding is that blockade of ghrelin signaling during stress is sufficient to prevent stress-related vulnerability to excessive fear. This raises the possibility that such a strategy might reduce or prevent the development of stress-sensitive affective disorders like PTSD during prolonged or extreme stress load. To date, very few studies have examined ghrelin or its receptor in the development of affective mental illness; however, polymorphisms in the ghrelin gene have been associated with panic disorder,[85] and serum ghrelin levels are higher in patients with treatment-resistant forms of major depressive disorder or panic disorder.[59] Although there are some non-HPA molecules that might be targeted in the treatment of stress-sensitive mental illnesses (such as brain-derived neurotrophic factor, tissue plasminogen activator or FKBP5; for review, see Mahan and Ressler[86]), the dysregulation of these molecules in rodent models of these diseases is brain region-specific. To effectively treat mental illness, pharmaceuticals for these molecules would need to cross the blood–brain barrier and act in a brain region-specific manner to minimize off-target side effects. Furthermore, there are no pharmaceuticals that can readily affect these molecules in humans. In contrast, because ghrelin is a peripheral hormone, it can be targeted using therapeutics that act in the periphery. Finally, many antighrelin treatments have already undergone rigorous safety testing in humans because of the putative role of ghrelin in the development of obesity.[87] Thus, the discovery that ghrelin plays a role in stress-related affective dysregulation reveals an especially attractive target for treating stress-sensitive mental disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

RMM conceived experiments, designed protocols, performed experiments and completed histology and data analyses for Figures 1–4 and Supplementary Figures S1, S2, S3c, and S4–S8. ABR performed experiments, histology and data analyses for Figures 5b–d and f–h. EL designed and performed experiments and completed data analysis for Supplementary Figures S3a and b. SSC conceived, designed, performed and analyzed the experiment in Supplementary Figure S10. KAG conceived and designed experiments, prepared GH virus, performed the experiment in Figures 5a and e–h and Supplementary Figure S9 and performed data analysis. RMM and KAG designed figures and wrote the manuscript.

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