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Central Ghrelin Resistance Permits the Overconsolidation of Fear Memory

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Abstract

Background—There are many contradictory findings on the role of the hormone ghrelin in aversive processing, with studies suggesting that ghrelin signaling can both inhibit and enhance aversion. Here, we characterize and reconcile the paradoxical role of ghrelin in the acquisition of fearful memories.

Methods—We used enzyme-linked immunosorbent assay (ELISA) to measure endogenous acyl-ghrelin and corticosterone at time points surrounding auditory fear learning. We used pharmacological (systemic and intra-amygdala) manipulations of ghrelin signaling and examined several aversive and appetitive behaviors. We also used biotin-labeled ghrelin to visualize ghrelin binding sites in coronal brain sections of amygdala. All work was performed in rats.

Results—In unstressed rodents, endogenous peripheral acyl-ghrelin robustly inhibits fear memory consolidation through actions in the amygdala and accounts for virtually all inter-individual variability in long-term fear memory strength. Higher levels of endogenous ghrelin after fear learning were associated with weaker long-term fear memories, and pharmacological agonism of the ghrelin receptor during the memory consolidation period reduced fear memory strength. These fear-inhibitory effects cannot be explained by changes in appetitive behavior. In contrast, we

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Author Contributions

Experiments were designed by ESH, LS, GL, SL, AM, NM, and KAG. Experiments were performed by ESH, LS, SL, AM, GL, EK, EL, XP, NM, and KAG. Data was analyzed by ESH, LS, SL, AM, BG, GL, EK, JY, EL, VSW, WS, XP, NM, and KAG. The manuscript was written by ESH and KAG. All authors edited the manuscript.

show that chronic stress, which increases both circulating endogenous acyl-ghrelin and fear memory formation, promotes both a profound loss of ghrelin binding sites in the amygdala and behavioral insensitivity to ghrelin receptor agonism.

Conclusions—These studies provide a new link between stress, a novel type of metabolic resistance, and vulnerability to excessive fear memory formation and reveal that ghrelin can regulate negative emotionality in unstressed animals without altering appetite.

Keywords

ghrelin; corticosterone; chronic stress; amygdala; fear; hunger

Introduction

Ghrelin, often called “the hunger hormone”, is an omnipresent circulating hormone that is synthesized and released by many organs; the stomach is the primary source of ghrelin in the bloodstream (1). In its acylated form (acyl-ghrelin), circulating ghrelin can cross the blood-brain barrier and bind to central ghrelin receptors (growth hormone secretagogue receptor 1a, or GHSR) (1). GHSRs are abundant in classic hypothalamic hunger regions (2). Also, ghrelin levels can be rapidly elevated within minutes during anticipatory hunger states (3) and administration of acyl-ghrelin to humans can stimulate food intake (4). However, GHSRs are widely distributed throughout the brain including brain regions not typically associated with hunger, such as the basolateral complex of the amygdala (BLA) (5), a brain region important for regulating valenced behavior, including fear. Additionally, acyl-ghrelin is tonically secreted at all times (6), not just at times in which food is expected. These findings suggest that acyl-ghrelin may have a broader role than simply modulating hunger or appetitive processing.

The presence of GHSR in the BLA suggests that ghrelin signaling may modulate fear, but the role of acyl-ghrelin in BLA-dependent fear memory has remained controversial. While some groups report that transient elevation of ghrelin signaling promotes the excitability of BLA neurons (7), others find that ghrelin decreases BLA excitability (5). Only two studies have directly assessed the role of ghrelin in the BLA, and those report that acute and chronic elevation of ghrelin enhance fear memory strength (8, 9). Here, we sought to resolve the role of endogenous acyl-ghrelin in BLA-dependent fear memory.

Methods and Materials

Blood was sampled from jugular catheters or tails at different time points surrounding auditory Pavlovian fear conditioning in rats. We used enzyme-linked immunosorbent assay (ELISA) to examine plasma acyl-ghrelin and corticosterone levels in these samples. In other experiments, we administered a ghrelin receptor agonist, a ghrelin receptor antagonist, or rat acylated ghrelin either systemically or intra-BLA. Following these manipulations, different behaviors were examined, including Pavlovian fear conditioning, fear recall testing, food consumption, or unconditional freezing. Some rats received chronic immobilization stress or handling; biotin ghrelin was used to assess ghrelin binding affinity in coronal brain sections

containing the BLA. Refer to the Supplementary Materials and Methods for procedural details.

Results

Using healthy, unstressed rats implanted with jugular vein catheters, we took blood samples at time points surrounding auditory Pavlovian fear conditioning (Figure 1A). We found that circulating acyl-ghrelin levels were not significantly altered by the brief (<3 min duration) fear conditioning paradigm used here (Figure 1B, upper), in contrast to corticosterone levels (Figure 1B, lower). Thus, while the expectation of food can rapidly elevate acyl-ghrelin levels within minutes (3), a brief fear conditioning experience, one form of acute stress exposure, does not. We previously reported that repeated, but not acute, stressor exposure elevates acyl-ghrelin levels measured 24 hr after stressor cessation (9); this extends these findings to show that acute stressor exposure does not change acyl-ghrelin on a shorter time scale.

We sought to determine whether acyl-ghrelin or corticosterone levels during fear learning determined subsequent long-term fear memory strength in rats. Long-term auditory fear memory strength was assessed two days following fear conditioning, a time point beyond the time in which short-term memory undergoes synaptic consolidation to form long-term memory (10). We computed 12 linear regressions with freezing as the dependent variable and acyl-ghrelin level at each time point (0 to 180 min) to determine whether acyl-ghrelin levels at individual time points around fear conditioning were related to long-term fear recall measured more than 24 hr later (see “Statistics” in Supp. Materials and Methods).

Acyl-ghrelin levels measured at 120 or 180 minutes after fear conditioning each individually accounted for a considerable amount of the variance in freezing behavior (R^2 = 0.94 and 0.93; Figure S1 A,B). Because the number of subjects for this experiment was low, we also computed the predicted residual sum of squares (PRESS) statistic for each regression. The PRESS statistic is a leave-one-out cross-validation method that provides an estimate of how well an equation will generalize to new data. The low PRESS values for the regressions against acyl-ghrelin at 120 and 180 minutes post-conditioning (Table S1) confirmed that the values at these time points were the most strongly correlated with freezing during the long-term memory recall test. We used Lasso regression (see “Statistics” in Supp. Materials and Methods, Figure S2A) to determine whether measurements of acyl-ghrelin and/or corticosterone levels at multiple time points could better explain freezing data than any single measurement. Two Lasso regressions were performed, with acyl-ghrelin or corticosterone levels from all time points as the independent variables. The best-fitting linear model included acyl-ghrelin plasma levels at both 120 and 180 minutes after fear conditioning (Figure 1C). Higher levels of endogenous circulating ghrelin across these time points robustly constrained auditory fear memory strength. Despite a substantial literature implicating glucocorticoids in memory formation (see (11) for review), none of the selected models retained corticosterone levels as a measurement to explain freezing behavior (Figure S2B, Table S1). Endogenous acyl-ghrelin levels did not correlate with shock reactivity during fear conditioning (Figure S1E) or with the HPA response induced by fear conditioning (Figure S1F). Because post-conditioning acyl-ghrelin plasma levels were

correlated with long-term auditory fear memory recall but not fear acquisition, this suggests that endogenous acyl-ghrelin might negatively regulate fear memory consolidation.

It could be argued that hunger and fear are incompatible states and thus compete with each other within neural circuits. This hunger/aversive behavior tradeoff has been noted in rodents in semi-naturalistic foraging environments (12) and has been described for a specialized subset of hypothalamic neurons (13). By this logic, high levels of acyl-ghrelin, which under some circumstances can promote hunger (14), may also facilitate exploration to increase the opportunity to find food. For example, increasing levels of acyl-ghrelin might facilitate motor hyperactivity, thereby decreasing freezing behavior. However, we did not observe correlations between gross motor activity and acyl-ghrelin levels across the subjects from Figure 1 (Figure S1G).

To further determine whether acyl-ghrelin was broadly related to fear memory strength, rather than simply to freezing behavior, we examined risk assessment behavior, in which the body remained immobile but the head was moved to scan the environment (see Supp. Materials and Methods), during the long-term auditory fear recall test (Figure 1D). Risk assessment behaviors are observed when threat levels are determined to be low or moderate (15) and are thought to be important for reducing defensive behaviors such as freezing when danger is no longer present (16). As perceived threat transitions from high to low levels, fear also shifts from high to low levels; increased risk assessment behaviors accompany this shift. No risk assessment behavior was observed in any rat prior to the tone onset of each fear recall test (data not shown), confirming that this behavior is specifically elicited during a fear state. We computed linear regressions with risk assessment behavior as the dependent variable and acyl-ghrelin level at each time point (0 to 180 min) to determine whether acyl-ghrelin levels at individual time points around fear conditioning were related to this second measure of fear memory strength. We found that acyl-ghrelin levels measured at 120 or 180 minutes after fear conditioning were also associated with earlier onset of vigilance behaviors (Figure 1D; Table S2), suggesting that rats with higher endogenous acyl-ghrelin levels more rapidly transition to a low fear state during the fear recall test. Thus, endogenous acyl-ghrelin was correlated with two distinct measures of fear memory strength. Collectively, these data suggest that plasma acyl-ghrelin levels are related to the strength of fear memories, rather than simple motor hyperactivity.

Because ghrelin can be elevated by hunger (17), it might be hypothesized that inter-individual variability in acyl-ghrelin arises when fear conditioning suppresses post-conditioning food consumption to different degrees in individuals in the hours following conditioning. However, acyl-ghrelin levels were similar before and after fear conditioning (Figure 1B) and fear conditioning did not suppress food consumption (Figure S1H). Thus, we hypothesized that baseline acyl-ghrelin levels are a stable and variable individual trait over time. Because postconditioning acyl-ghrelin levels were negatively correlated with long-term fear memory, and fear conditioning did not substantially alter acyl-ghrelin levels (Figure 1B), we further hypothesized that pre-conditioning acyl-ghrelin levels would also correlate with fear memory. To assess this, we conducted a second experiment in which we measured circulating acyl-ghrelin levels in unoperated rats at least one week prior to auditory Pavlovian fear conditioning (Figure 2A). We again observed that plasma acyl-

ghrelin levels exhibit a statistically significant negative association with long-term auditory fear memory (Figure 2B). This suggests that measuring acyl-ghrelin levels prior to an aversive experience might serve as a predictive biomarker for long-term fear memory strength.

Because enhanced consolidation of fearful memories is thought to underlie the development of some trauma and stress-related disorders such as post-traumatic stress disorder (PTSD), interventions that reduce the consolidation of fear memories represent a promising strategy to prevent the development of these disorders (18). Harnessing the power of an endogenous fear-inhibitory system such as ghrelin is especially promising because both ghrelin and pharmacological ghrelin receptor agonists have strong safety profiles in human subjects (19–22), including no reported side effects on other forms of memory. To determine whether fear memories can be further inhibited by artificially boosting the effects of endogenous acyl-ghrelin, we administered ibutamoren mesylate (IBU), a ghrelin receptor agonist (19), or vehicle (VEH) immediately after auditory Pavlovian fear conditioning (Figure 3A, upper panel). The systemic administration of IBU produced significantly lower levels of long-term auditory fear memory (Figure 3A, lower panel); the difference between the VEH and IBU groups was not observed during fear conditioning, which was prior to injection. Similar results were observed when acyl-ghrelin was infused directly into the BLA two hours after auditory fear conditioning (Figure 3B) or when IBU was administered either systemically (Figure S3A, lower panel) or into the BLA (Figure S3B, lower panel) prior to auditory fear conditioning: long-term auditory fear memory was inhibited. Systemic administration of a ghrelin receptor antagonist after fear conditioning produced the opposite effect (Figure S3C): long-term auditory fear memory was enhanced. Critically, although the dose of the ghrelin receptor agonist used here can exacerbate food consumption following acute food deprivation (Figure S3D), it did not stimulate hunger in the memory consolidation period following fear conditioning (Figure 3C; Figure S3E), during which rats were not food deprived. This is important because overt hunger could interfere with fear memory formation in a number of ways that are not directly related to the consolidation of associative fear memory, including the promotion of food-seeking behaviors that compete with freezing (12, 23), or the bias of attention away from fear cues and towards the detection of food (24). Our data reveal that enhanced ghrelin signaling decreases fear memory strength in the absence of possible confounds of hunger.

The half-lives of IBU and acyl-ghrelin are brief [~6 hours for IBU (25) and ~ 30 min for acyl-ghrelin (26)]. Thus, post-conditioning administration of these substances (Figure 3) can only impact fear memory consolidation, and not sensory processing or memory retrieval or performance during the fear recall test 48 hr later. Additionally, we found that pre-retrieval administration of IBU did not impact auditory fear memory strength (Figure S4) revealing that elevated ghrelin signaling during fear recall does not impact fear memory retrieval, fear expression, or the processing of auditory stimuli. Similarly, we found that elevated ghrelin signaling did not affect unconditional freezing (Figure S5); this suggests that ghrelin does not interfere with the motor systems required to express a freezing response. Collectively, our results support the idea that ghrelin signaling regulates fear memory consolidation, rather than other processes which impact fear memory encoding and recall, or fear expression.

It may be surprising that higher levels of GHSR signaling are associated with weaker fear memories. In rats exposed to chronic stress, elevated acyl-ghrelin (27) was associated with enhanced fear memory formation (9). In contrast, here we show that higher plasma levels of acyl-ghrelin are associated with weaker fear memories in rats lacking a history of repeated stressor exposure. To explain this apparent discrepancy, it should be noted that previously reported levels of acyl-ghrelin observed after *chronic* stress (9) tend to be significantly higher than the highest physiological levels observed in either the *absence* of prolonged, intense stress or after *acute* stress (Figure 1B). Second, similar opposing effects of ghrelin have been noted for anxiety (28), a different form of aversive behavior, but the mechanism by which this occurs remains unknown. To explain the paradoxical effects of ghrelin in unstressed versus chronically stressed rodents, we hypothesized that stress-induced elevation of acyl-ghrelin promotes central ghrelin resistance, where cells compensate for stress-induced upregulation of signaling through ghrelin-mediated pathways by downregulating expression of GHSR.

To assess this, rats were sacrificed one day following the last day of a two week period of either chronic immobilization stress or handling (Figure 4A). Binding sites for acyl-ghrelin in the BLA were identified with biotin-labeled acyl-ghrelin (Figure 4B); signal was absent in sections incubated without biotin-labeled acyl-ghrelin (Figure S6). Ghrelin binding was dramatically decreased in the BLA of stressed rats compared to that of unstressed rats (Figure 4C). In unstressed rats, the availability of ghrelin binding sites exhibited a negative correlation with endogenous acyl-ghrelin levels measured 15 days prior to sacrifice (Figure 4D); this correlation was lost in stressed rats, who exhibited levels of ghrelin binding that were uniformly lower than those observed in unstressed rats (Figure 4D).

To determine whether the decrease in ghrelin binding sites in the BLA of chronically stressed rats exerts a functional impact, rats received either chronic immobilization stress or handling for two weeks followed by auditory fear conditioning (Figure 4E). IBU, at the dose shown to reduce fear memory strength in unstressed rats (Figure 3), or VEH was injected immediately after conditioning and long-term auditory fear memory was assessed two days later. While IBU reduced long-term auditory fear memory in unstressed rats, it had no effect on conditional freezing in stressed rats (Figure 4F), supporting the idea that chronic stress promotes functional ghrelin resistance. This contributes to an emerging literature showing that ghrelin resistance in brain circuits can promote dysfunction. For example, functional ghrelin resistance, defined by a behavioral insensitivity to ghrelin, has been observed in reward pathways (29) and in the hypothalamus (30), but these forms of ghrelin resistance were induced by low levels of circulating acyl-ghrelin and no mechanism was demonstrated. Our study is the first to report a specific mechanism (loss of ghrelin receptor binding) for central ghrelin resistance following high circulating acyl-ghrelin levels.

Discussion

We report here a robust correlation between individual levels of circulating ghrelin and long-term auditory fear memory in rats lacking a history of repeated exposure to intense stress; this may reflect a hormetic benefit of ghrelin. In addition to its fear-inhibiting effects, ghrelin exerts other beneficial effects, including enhanced longevity during caloric

restriction (31), dendritic growth (32), improved cardiac function (33), and increased slow-wave sleep (34). These effects have been studied by comparing engineered mouse strains with different levels of acyl-ghrelin, or by applying exogenous ghrelin, and it remains to be seen whether individual variation in acyl-ghrelin correlates with differences in these measures, as we report here for fear.

Historically, many molecules that regulate fear memory consolidation have been identified because they show a selective change in activity during the consolidation window and play an instructive role in long-term memory (35–39). These instructive molecules regulate consolidation through phasic, transient changes in signaling. However, there is increasing recognition that molecules [such as Fibroblast Growth Factor 2 (40)] and processes (such as epigenetics) can also play a *permissive* role in memory consolidation. Permissive influences on memory consolidation could be mediated, for example, by structural changes, alterations in the availability or activity of plasticity-related proteins, or changes in receptor availability, which can occur prior to learning. In these cases, the permissive molecules are not a trigger for memory consolidation *per se*, and the activity of such molecules may not be altered by the acquisition of plasticity itself. Thus the absence of transient, stress-induced changes in acyl-ghrelin levels during fear memory consolidation that we report here should not be interpreted as evidence that acyl-ghrelin cannot be involved in consolidation. Rather, such a finding suggests that tonic signaling through ghrelin pathways is a modulator of consolidation, rather than phasic changes in signaling. The importance of tonic over phasic signaling has been noted for other neuromodulatory systems, such as the role of dopamine in determining motivational vigor (41, 42).

The tonic actions of ghrelin could impact fear memory consolidation through multiple mechanisms. Because acyl-ghrelin is omnipresent in blood, there is a constant degree of direct, ligand-dependent signaling through GHSR1a homodimers (43) which could synergize with memory consolidation triggered by learning. Such synergistic signaling could be further enhanced by administration of ibutamoren mesylate (Figure 3). In addition, GHSR1a homodimers exhibit a high level of ligand-independent constitutive activity (44), and the activation of intracellular pathways via this mechanism could, in theory, impact memory consolidation. In addition to these signaling pathways, ghrelin may have additional effects mediated via heterodimers with other neuromodulatory receptors, including dopamine (45, 46) and serotonin (45) receptors. Though GHSR1a can modulate these other receptors in the absence of acyl-ghrelin, rapid ligand-dependent removal of GHSR1a from the membrane (47) could decrease the availability of GHSR1a for heterodimerization, and thus impact GHSR1a-dependent modulation of dopamine, serotonin and other neuromodulators during memory consolidation. The administration of ibutamoren mesylate could enhance ligand-dependent internalization of GHSR1a and thus deepen the impact of endogenous acyl-ghrelin on heterodimer-dependent signaling during memory consolidation. The complexity of signaling pathways induced by ghrelin poses a formidable challenge to understanding precisely which pathways may contribute to fear memory consolidation, but this remains a rich topic for future studies.

Our results show that the BLA is one critical site by which systemic ghrelin receptor agonists can inhibit fear memory consolidation. However, many other brain regions outside

the BLA express GHSR (2), and many other brain regions outside the BLA participate in fear memory (48). It is possible that endogenous ghrelin may inhibit fear memory consolidation via actions in some of these brain regions, and that ghrelin resistance in some of these brain regions may also contribute to stress-related enhancement of fear. This is also an important topic for additional studies.

While a few previous studies have examined the role of ghrelin in fear-related tasks, these are difficult to directly compare to the findings here. In one study, intra-amygdala infusions of ghrelin were shown to enhance avoidance memory (8), seeming to conflict with our observations reported here. However this study targeted the central nucleus of the amygdala and the species of ghrelin was not indicated. Additionally, the surgical procedure to implant cannulae in the brain might have served as a chronic stressor which induced the loss of ghrelin-mediated inhibition in the amygdala. In a different study, transgenic mice with a knockout of the ghrelin 1a receptor exhibited a selective impairment in contextual fear memory levels measured one month after fear conditioning (49). One potential caveat to interpreting these findings is that the developmental knockout may have led to compensation in ghrelin receptor-expressing brain regions such as the BLA. Additionally, it is not clear what memory-related process was affected, and thus what brain regions might be implicated. For example, the deficit in contextual fear at 30 days after the first extinction session could reflect enhanced fear extinction in the ghrelin knockout mice, or impaired fear incubation. Without further data, these findings are difficult to interpret in light of our present results.

Interestingly, post-training levels of norepinephrine in the BLA show considerable variability across individuals and are a positive predictor of long-term aversive memory strength (36). Thus, while norepinephrine accelerates the consolidation of aversive memory and confers susceptibility to “overconsolidation” of aversive experiences, ghrelin provides a previously unrecognized opposing force which confers resilience to the overconsolidation of aversive experiences. Post-training glucocorticoid levels also modulate fear memory strength (37, 50, 51), an effect we replicated here.

Our findings are consistent with previously published results showing that repeated stress (9, 27), but not acute stress (9), increases circulating acyl-ghrelin. Additionally, while the single housing of our rodents may have served as a chronic, low intensity stressor in our “unstressed” groups, it is clear that prolonged immobilization stress imposed an additional source of stress which served to drive further biological changes. It is unclear why repeated stress elevates acyl-ghrelin (9, 27), but acute stress does not elevate acyl-ghrelin either in the hours following stress (Figure 1B) or 24 hr later (9). One possibility is that ghrelin may be elevated in anticipation of an energy deficit (52), and repeated stress may be more likely to produce a state of “wear and tear” in which bodily resources are depleted (53). In this regard, acyl-ghrelin may be unusual as a biomarker of stress because it may be selectively engaged during times of allostatic load (54). The mechanisms by which acyl-ghrelin is elevated after repeated stress represent a fascinating topic for future studies.

Our findings support the idea that the excessive fear observed in chronically stressed rats [Figure 4F and (9)], who also have high circulating ghrelin levels (9), likely arises, in part, from stress-induced loss of an inhibitory signal in the BLA. Our findings also suggest that

the enhancement of fear that follows either chronic delivery of a ghrelin agonist or chronic intra-BLA infusion of ghrelin in rats without a history of stress exposure (9) arises because these treatments promote central ghrelin resistance, leading to insensitivity to the fear-inhibiting effects of endogenous ghrelin; in contrast, unstressed rats that receive a single dose of a ghrelin agonist (Figure 3A) or a single intra-BLA infusion of ghrelin (Figure 3B) are sensitive to the fear-inhibitory effects of these treatments because ghrelin resistance is absent. One remaining question is whether ghrelin resistance can be induced in brain circuits that support other stress-sensitive behaviors. For example, chronically elevated ghrelin is associated with hyperaggression in mice (55).

Although chronically stressed rats have high levels of acyl-ghrelin and elevated fear, it is not correct to infer that acyl-ghrelin stimulates or promotes fear in stress-exposed animals. Insofar as ghrelin represents an endogenous “brake” for fear memory strength, chronic stress removes this inhibitory force by downregulating the binding of acyl-ghrelin to its receptors. Chronic stress may also alter other intracellular signals which can act as “accelerants” for fear, such as glutamate receptors (56) and others (57), but there is no direct evidence that ghrelin itself acts in this manner after stress exposure.

It may seem counterintuitive that ghrelin can modulate fear memory consolidation without changing hunger or appetitive processing because ghrelin was first characterized as a hunger hormone and hunger can exert potent influences on emotional tone (13, 58). We deliberately utilized doses of a ghrelin agonist or acyl-ghrelin below the threshold for inducing hunger (see Drug Preparation section of Supp. Materials and Methods) because hunger can produce behaviors that compete with the expression of fear behaviors (12, 23), and hunger and aversive behavior also interact at the neural circuit level (13). Thus, while hunger and fear can interact, we examined a role for ghrelin in fear in the absence of potential confounds induced by hunger.

Additionally, endogenous acyl-ghrelin signals hunger as a large, acute spike over “background” acyl-ghrelin levels (3, 59). This suggests that hypothalamic hunger circuits are relatively insensitive to acyl-ghrelin, as large spikes in acyl-ghrelin are required to drive the subjective feeling of hunger. In contrast, we report here that the ability of the BLA to modulate fear memory consolidation is sensitive to tonic, background levels of acyl-ghrelin, a factor which has not been linked to hunger. Here, we show that these levels of ghrelin have a robust correlation with fear memory strength without impacting exploratory behavior, food consumption or body weight. We suggest that, in animals without a history of prolonged stressor exposure, ghrelin may serve a permissive role for exploration and consummatory behaviors, reducing fear which can compete with such behaviors. However, other hormones and activity in neural circuits must provide the driving force behind exploratory and consummatory behaviors because relatively high tonic levels of plasma acyl-ghrelin are not sufficient to evoke these behaviors in unstressed animals.

There are no studies examining the effects of acyl-ghrelin or ghrelin-based drugs on fear memory in humans, yet an abundance of data suggests that ghrelin receptor agonists are well-tolerated (19, 20, 22) with increased appetite, transient mild edema and transient muscle pain as the most common adverse effects; no effects on memory were noted. In

general, ghrelin receptor agonists are believed to have pro-cognitive effects (60), making it more remarkable that ghrelin constrains memory consolidation and reduces auditory fear memory strength.

Our results indicate that ghrelin receptor agonism inhibits BLA-dependent auditory fear while generally enhancing hippocampus-dependent contextual fear in rats lacking a history of prolonged stressor exposure (61). The latter effect is consistent with other studies showing that acute ghrelin receptor agonism enhances glutamatergic neurotransmission and plasticity in the hippocampus (62, 63). It is unclear why ghrelin-mediated signaling in the amygdala favors the reduction, rather than the enhancement, of fear memory. There are several potential mechanisms by which this could occur, including ghrelin-mediated excitation of inhibitory interneurons (Figure S7), ghrelin-mediated excitation of glutamatergic projection neurons in the BLA that selectively inhibit fear memories (64), and recruitment of inhibitory G protein-mediated signaling cascades (65). Future work will undoubtedly dissect the contribution of these complex mechanisms. Regardless of the mechanism by which ghrelin regulates memory consolidation, our findings contribute to an emerging literature supporting the idea that a neuromodulator may have very different effects on plasticity in different neural circuits.

The opposing effects of ghrelin receptor agonism on hippocampus- and amygdala-dependent fear memories make this a promising candidate system for the treatment of PTSD in humans; PTSD is associated with hyperactivation of the amygdala (66) but impaired contextual processing (67). Thus, ghrelin receptor agonism following trauma might constrain amygdala hyperactivation while promoting hippocampus-dependent contextual processing. The enhancement of endogenous mechanisms for inhibiting fear memory consolidation represent an attractive avenue for preventing PTSD following trauma in humans (18). Two other endogenous substances are known to inhibit fear memory formation: the inhibitory neurotransmitter GABA and the endogenous opioid β-endorphin. These substances and acyl-ghrelin may be thought of as “resilience factors” because higher levels of these endogenous substances constrain the impact of trauma, potentially preventing the emergence of PTSD. Unlike opioid or GABA receptor agonists, ghrelin agonists are not addictive and therefore may represent a less risky but equally promising intervention in trauma-exposed humans at risk for PTSD. However, our work also highlights that a history of stress exposure promotes ghrelin resistance in the amygdala. This suggests that in humans with a significant stress burden prior to trauma, higher doses of ghrelin receptor agonists may be required to attain therapeutic efficacy in fear memory reduction. Alternatively, ghrelin resistance may need to be reduced, perhaps by lowering circulating ghrelin levels, before therapeutic efficacy may be achieved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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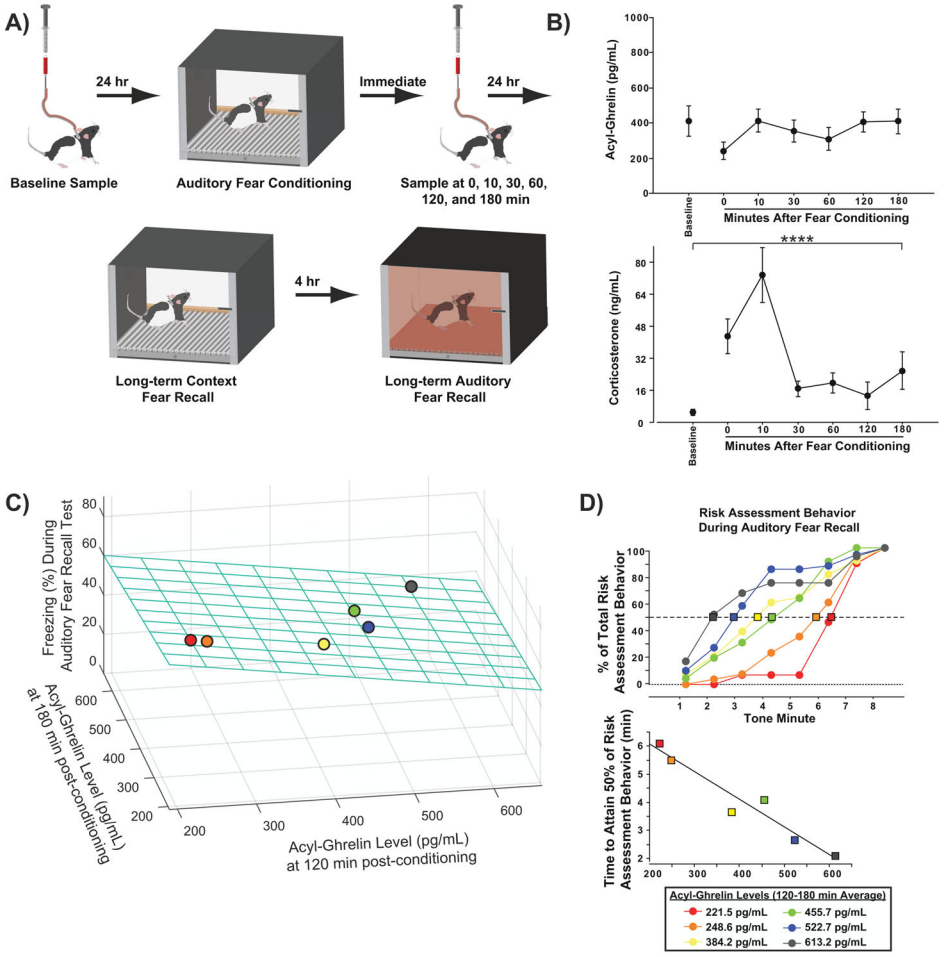

Figure 1. Post-conditioning acyl-ghrelin levels are a negative predictor of long-term fear memory strength

A) Experimental design (n = 6). Panels (B)-(D) depict data from these rats. B) Acyl-ghrelin levels were not altered by fear conditioning [time: $F(5,30) = 1.16$, $p = 0.35$]. In contrast, corticosterone levels were rapidly and transiently elevated following fear conditioning [time: $F(5,30) = 9.15$, $p < 0.0001$]. A long-term auditory fear recall test was conducted two days later, and freezing (C) and risk assessment behavior (D) were measured during an 8 min tone presentation. Each rat is represented by the same color in panels (C) and (D). C) The best fit plane from a regularized linear regression, using the Lasso method, of freezing (%) predicted by ghrelin levels after fear conditioning is shown. The best fit plane is shown in green; each point represents one rat. The linear model coefficients retained by the Lasso method were [0 0 0 0 −0.053 −0.029] for ghrelin levels at *minutes after fear conditioning* = [0 10 30 60 120 180] respectively, with an intercept of 89.5 pg/ml. When the Lasso method was used as a model selection technique, ghrelin levels at time points 120 minutes and 180 minutes were retained for a subsequent multivariate linear regression. That regression produced coefficients of [−0.075 −0.047] for ghrelin levels at *minutes after fear conditioning* = [120 180] respectively, an intercept of 105.67 pg/ml, R-squared = 0.96, p-value for the model= 0.007, root mean squared error (RMSE) = 4.59, and predicted residual sum of

squares statistic (PRESS) = 248. D) *Upper,* A cumulative percentage plot for risk assessment behavior in individual rats during the long-term auditory fear recall test is shown. Square points represent the time at which 50% of risk assessment behavior was shown for each rat, estimated from sigmoidal curves fit to each rat's data. *Lower*, The plot depicts the average level of acyl-ghrelin versus the time at which 50% of risk assessment behavior is shown for each rat. Error bars represent ±SEM. *p < 0.05, *** p < 0.001, ****p < 0.0001

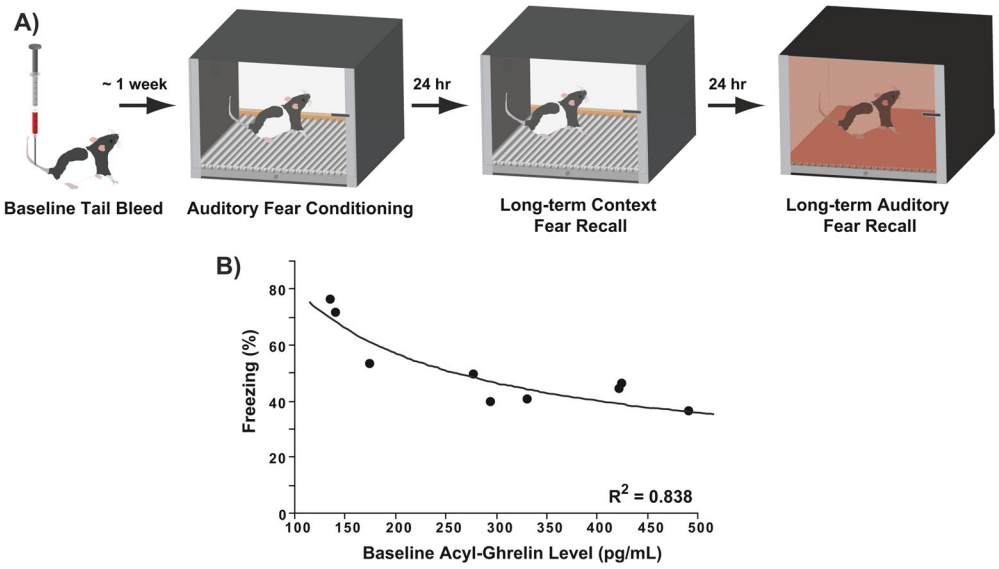

Figure 2. Pre-conditioning baseline acyl-ghrelin levels are a negative predictor of long-term fear memory strength

A) Experimental design (n = 9). To examine correlations between long-term fear memory and acyl-ghrelin levels measured in blood samples collected at least one week prior to fear conditioning, lateral tail vein sampling was used on unoperated rats. To avoid the influence of stress from this procedure on behavior, additional baseline blood samples were not collected. B) The baseline acyl-ghrelin levels were negatively associated with subsequent long-term auditory fear memory (averaged across the 8 min tone test) (R^2 = 0.838 for a power model; R^2 = 0.685 for a linear model).

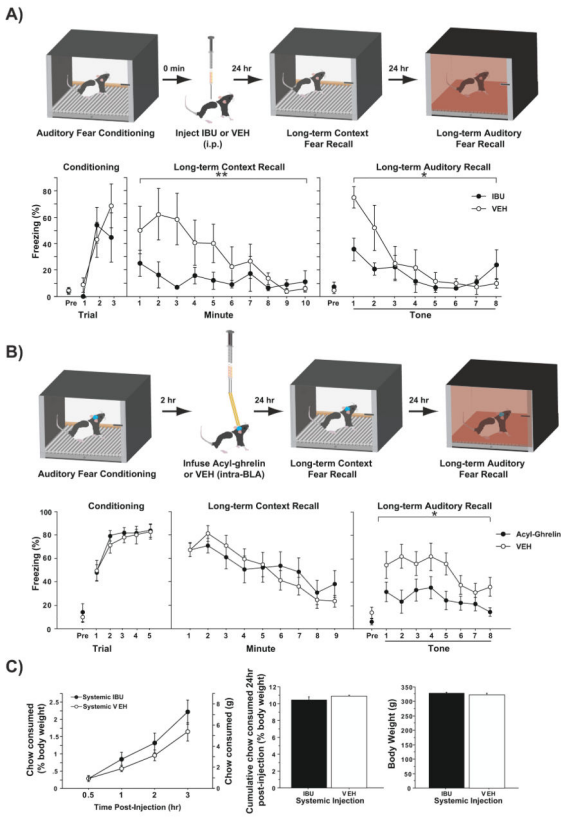

Figure 3. Enhancing the effects of endogenous ghrelin during the consolidation window further constrains fear memory strength

A) *Upper*, Experimental design. *Lower*, Systemic administration of a ghrelin receptor agonist (IBU; 0.5mg/mL) following fear conditioning significantly impaired both long-term contextual fear memory [group X time interaction: $F(9,72) = 4.28$, $p < 0.01$] and long-term auditory fear memory [group: $F(7,56) = 2.48$, $p < 0.05$] without affecting fear acquisition [group: $F(1,8) = 0.29$, $p = 0.61$]. (n = 5/group) B) *Upper*, Experimental design. *Lower*, Intra-BLA administration of acyl-ghrelin (0.05 nmol in 0.5μl per BLA) following fear conditioning significantly impaired long-term auditory fear memory [group: $F(1,16) = 5.48$, $p < 0.05$] without affecting fear acquisition [group: $F(1,16) = 0.18$, $p = 0.68$] (n = 9/group). C) In a separate group of animals that received fear conditioning and injections as described in panel (A),systemic administration of IBU did not impact food consumption either shortly following injection (left panel; injection: $F(1,12) = 1.58$, $p = 0.23$; injection X time interaction: $F(3,36) = 1.39$, $p = 0.26$) or cumulatively, over the 24 h following injection (middle panel; injection: $F(1,12) = 1.23$, $p = 0.29$). Body weight did not differ between the two groups (right panel; injection: $F(1,12) = 0.39$, $p = 0.54$) (n = 6–8/group). Error bars represent ±SEM. *$p < 0.05$, **$p<0.01$

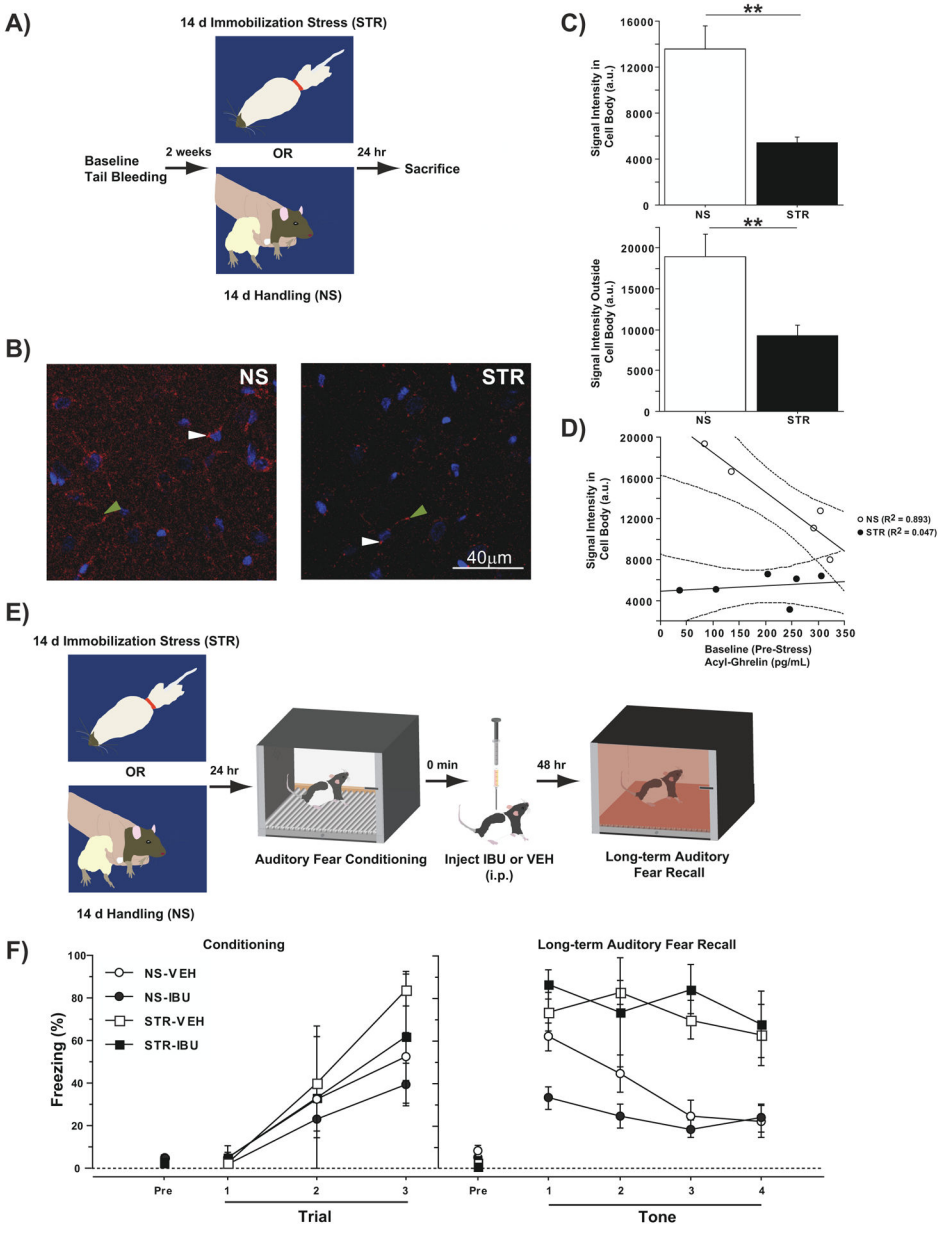

Figure 4. Chronic stress decreases ghrelin binding in the amygdala and renders animals insensitive to the fear-reducing effects of a ghrelin receptor agonist

A) Experimental design. B) Representative confocal images (20X) of biotinylated ghrelin binding (red puncta) in the BLA of an unstressed (NS) or chronically stressed (STR) rat. Blue signal represents nuclear DAPI staining. White arrowheads indicate representative ghrelin binding in cell bodies. Green arrowheads point to staining present in the inter-neuronal cell body spaces. C) Chronic stress significantly decreases the binding of biotinylated ghrelin in the nuclei (upper panel; group: $F(1,10) = 9.42$, $p < 0.05$) and processes (middle panel; group: $F(1,10) = 4.98$, $p < 0.05$) of the BLA (n = 5–6/group). D) In the unstressed animals from panel (C), there is a relationship between baseline acyl-ghrelin plasma levels and biotinylated ghrelin binding in the BLA: higher levels of circulating acyl-

ghrelin are correlated with lower levels of binding. This relationship is lost in animals that experience chronic stress. E) Systemic administration of a ghrelin receptor agonist (IBU) prior to fear conditioning (n = 8–9/group) significantly impaired long-term auditory fear memory in unstressed animals but not those exposed to chronic stress (group X time interaction: $F(9,93) = 1.99$, $p < 0.05$). No group differences were observed during fear conditioning (group X time interaction: $F(6,62) = 0.82$, $p = 0.56$). Error bars represent ±SEM. *$p < 0.05$