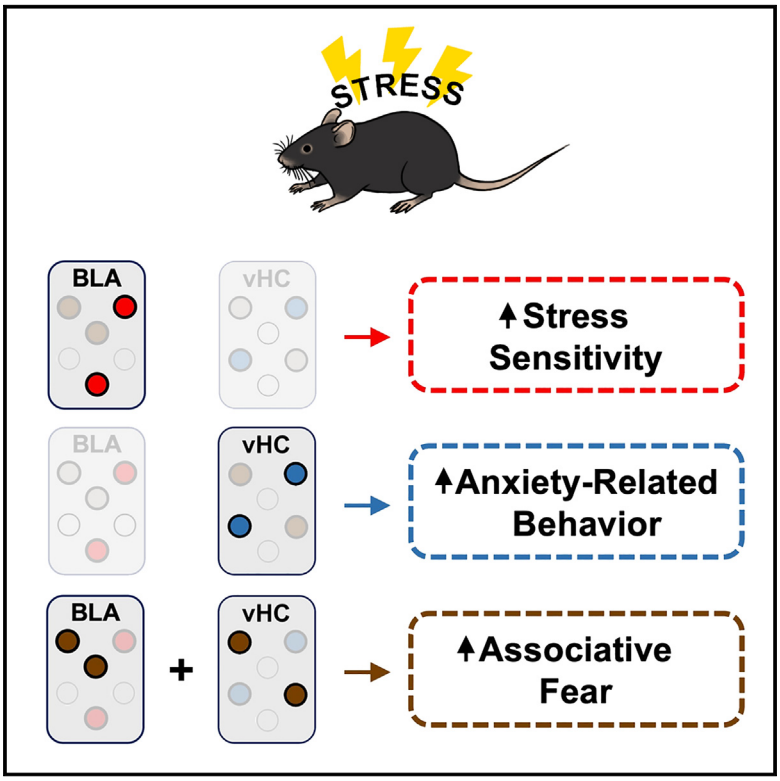
Cell Reports

Article

Dissociable contributions of the amygdala and ventral hippocampus to stress-induced changes in defensive behavior

Graphical abstract



Authors

Zachary T. Pennington, Alexa R. LaBanca, Patlapa Sompolpong, ..., Ian Maze, Tristan Shuman, Denise J. Cai

Correspondence

zachary.pennington@mssm.edu (Z.T.P.), denisecai@gmail.com (D.J.C.)

In brief

Impacts of stressful life events are often attributed to memories of stress-associated cues. Pennington et al. demonstrate that increased anxiety-related behavior and sensitivity to novel stressors resulting from a stressful life event are independent of these memories. They find that the amygdala and hippocampus contribute differently to these behaviors.

Highlights

- Stress induces changes in both associative and non-associative defensive behaviors
- Increases in stress sensitivity and anxiety-related behavior are non-associative
- Basolateral amygdala (BLA) is required for heightened stress sensitivity
- Ventral hippocampus (vHC) is required for increased anxiety-related behavior

Pennington et al., 2024, Cell Reports *43*, 114871
November 26, 2024
https://doi.org/10.1016/j.celrep.2024.114871

CellPress

Cell Reports

CellPress
OPEN ACCESS

Article

Dissociable contributions of the amygdala and ventral hippocampus to stress-induced changes in defensive behavior

Zachary T. Pennington,[1,*] Alexa R. LaBanca,[1] Patlapa Sompolpong,[1] Shereen D. Abdel-Raheim,[1] Bumjin Ko,[1] Zoe Christenson Wick,[1] Yu Feng,[1] Zhe Dong,[1] Taylor R. Francisco,[1] Madeline E. Bacon,[1] Lingxuan Chen,[1] Sasha L. Fulton,[1] Ian Maze,[1,2,3] Tristan Shuman,[1] and Denise J. Cai[1,4,*]

[1]Nash Family Department of Neuroscience, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
[2]Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
[3]Howard Hughes Medical Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
[4]Lead contact
*Correspondence: zachary.pennington@mssm.edu (Z.T.P.), denisecai@gmail.com (D.J.C.)
https://doi.org/10.1016/j.celrep.2024.114871

SUMMARY

Stress can have profound consequences on mental health. While much is known about the neural circuits supporting associative memories of stressful events, our understanding of the circuits underlying the non-associative impacts of stress, such as heightened stress sensitivity and anxiety-related behavior, is limited. Here, we demonstrate that the ventral hippocampus (vHC) and basolateral amygdala (BLA) support distinct non-associative behavioral changes following stress. Inhibiting stress-induced protein synthesis in the BLA blocked subsequent increases in stress sensitivity but not anxiety-related behaviors. Conversely, inhibiting stress-induced protein synthesis in the vHC blocked subsequent increases in anxiety-related behavior but not stress sensitivity. Inhibiting neuronal activity in the BLA and vHC during the assessment of stress sensitivity or anxiety-related behavior recapitulated these structures' dissociable contributions to defensive behavior. Lastly, blocking the associative memory of a stressor had no impact on stress-induced changes in anxiety-related behavior. These findings highlight that multiple memory systems support the long-lasting effects of stress.

INTRODUCTION

Animals display evolutionarily conserved defensive responses in immediate response to stressful and life-threatening events, including changes in heart rate and respiration, stress hormone release, and behavioral initiation of fight, flight, and freezing.[1–6] If sufficiently strong, stressful events can also instantiate persistent changes in how animals interact with their environment. Perhaps most extensively studied are associative fear responses, in which animals engage in defensive behaviors such as freezing and/or flight when re-exposed to environmental cues present at the time of the initial stressful experience.[1,7–12] However, after severe stress, animals also display alterations in foraging and exploration in uncertain environments[3,5,13,14] (often referred to as anxiety-related behavior), as well as heightened responses to future stressful events.[14–17] These long-lasting defensive behavioral changes are fundamental to anxiety disorders, which include fear of stress-related cues, heightened stress responses, and reduced environmental engagement, and are frequently predated by the experience of severe stress.[18–21]

It is often assumed that many of the defensive behavioral changes observed in the aftermath of stress are fundamentally associative in nature—animals could be responding to either cues that were directly present at the time of stress or stimuli resembling these cues to some degree (i.e., stimulus generalization).[22–30] For example, it is well documented that startle responses are potentiated by the presence of associative fear cues,[31–34] suggesting that associative stimuli may drive heightened responses to aversive events after stress. Moreover, it is possible that following stress, alterations in exploration in anxiety-related behavior tests such as the elevated-plus maze could be accounted for by shared features with the environment in which the stressor took place. Lastly, several reports document altered associative fear learning and generalization in humans with anxiety disorders.[23,24,33,35–37] In light of these findings, broad emphasis has been placed on associative learning processes governing the lasting consequences of stress. However, the explanatory reach of an associative framework has its limits. Pre-weanling rodents incapable of forming associative fear memories have nevertheless been found to display increased anxiety-related behavior in adulthood, as well as heightened responses to subsequent aversive experiences.[14] Moreover, extinguishing fear of stress-associated cues does not necessarily mitigate sensitized responses to new stressors.[15,38,39] These findings highlight the persistence of some stress-induced

behavioral phenotypes despite weak associative fear, indicating a potential dissociation. As such, it could be the case that multiple memory systems—associative and non-associative—support the enduring consequences of stress on defensive behavior. However, a direct biological dissociation of such memory systems has remained elusive. If discovered, this would have broad implications for the treatment of anxiety disorders, potentially explaining why treatments focused on associative processes are ineffective in some individuals.[40–42]

Here, we explore the contributions of stress-induced plasticity within the ventral hippocampus (vHC) and basolateral amygdala (BLA) to the enduring impacts of stress on associative and non-associative defensive behaviors. Neuronal activity within both the BLA and vHC is well known to regulate defensive behaviors.[43–55] However, whether stress-induced plasticity within these structures acts in concert to support a common defensive behavioral process or whether they support distinct defensive behavioral changes is unclear. Furthermore, a direct comparison of these structures' contribution to associative and non-associative defensive processes is lacking. We find that plasticity and neuronal activity within the BLA and vHC support separate non-associative defensive behavioral changes in response to stress and that the interaction of these structures is not essential to the expression of these same stress-induced defensive behavioral changes. These findings demonstrate unique functions of these structures and support the view that multiple memory circuits underlie stress-induced defensive behavioral changes.

RESULTS

Acute severe stress produces multiple lasting changes in defensive behavior

We first sought to establish a behavioral protocol in which a single acute stressor produces lasting changes in multiple defensive behaviors, adapting a prior model that has been used extensively in mice and rats[14,15,56–58] (Figure 1A). Animals were placed in a distinct environment where they received 10 footshocks during a 10-min period (Figure 1B, "trauma" [T]) or in the same environment but did not receive footshocks ("no trauma" [NT]). A week later, multiple defensive behaviors were assessed. To assess associative fear, animals were returned to the trauma environment (trauma recall). As expected, trauma-exposed animals spent a large amount of time freezing (Figure 1C; trauma: $F_{1,52} = 121.6$, $p < 0.001$). In the light-dark box, an exploratory test that captures rodents' natural avoidance of well-lit places and is sensitive to anxiolytics,[59,60] trauma-exposed animals showed increased anxiety-related behavior, reflected in more time spent in the dark side of the light-dark box (Figure 1D; trauma: $F_{1,52} = 19.7$, $p < 0.001$). Lastly, we assessed the animals' stress sensitivity by placing the animals in a novel environment, in which they showed virtually no freezing at baseline (Figure 1E, left; trauma: $F_{1,52} = 2.6$, $p = 0.11$). A loud auditory startle stimulus was then presented. When returned to this environment the next day, trauma-exposed animals showed substantially more freezing and evidence of stress sensitization (Figure 1E, right; trauma: $F_{1,52} = 16.1$, $p < 0.001$). Importantly, we demonstrate that all of these defensive behavioral changes—in associative fear, anxiety-related behavior, and stress sensitization—are proportional to the magnitude of the initial trauma (Figure S1). Additionally, although sex differences are common among anxiety disorders,[61,62] we found no behavioral differences between male and female mice in the dependent variables examined (Figure S1). Lastly, although stress sensitization is often termed stress-enhanced fear learning,[15] learning curve analyses revealed that enhanced learning likely reflects heightened sensitivity to aversive stimuli as opposed to an enhanced learning rate (Figure S2).

Next, as a preliminary means of addressing if these defensive behaviors convey information about unique biobehavioral processes, we correlated these phenotypes in a large group of trauma-exposed animals (vehicle-treated control animals in Figures 2F–2J), as high inter-phenotype correlations would suggest shared biological origins. We found relatively small correlations between behavioral tests, with large amounts of variance in each being unexplained by the others (Figures 1F–1H; trauma recall and light-dark: $R^2 = 0.04$, $p = 0.19$; trauma recall and novel stressor: $R^2 = 0.19$, $p < 0.01$; light-dark and novel stressor: $R^2 = 0.13$, $p = 0.32$). Though each of these measures is likely subject to imperfect test-retest reliability, these findings nevertheless suggest that these phenotypes may be independently governed.

Stress-induced protein synthesis in the BLA and vHC produces distinct changes in non-associative defensive behavior

In order to assess how stress-induced plasticity supports persistent changes in defensive behavior, we utilized post-stress administration of the protein synthesis inhibitor anisomycin, as protein synthesis is known to support the consolidation of many forms of memory and regulate synaptic plasticity.[63–69] Furthermore, because manipulations of protein synthesis can be done after a learning experience, they provide a means of disrupting the consolidation of a memory without altering its initial encoding.

To validate that the emergence of the observed defensive behavioral changes is indeed supported by stress-induced protein synthesis, we first assessed the effects of systemically administering anisomycin at various time points after trauma (Figures 2B–2E). Animals underwent trauma and were given anisomycin either immediately after trauma (T: ani (0h)) or 48 h later (T: ani (48h)). Alternatively, animals underwent trauma but were injected with vehicle (T: veh), or they were placed in the same environment but did not receive trauma and were treated with vehicle (NT: veh). During the initial trauma, groups receiving the trauma did not differ in their level of freezing, indicating no pre-existing differences existed between groups (Figure 2B; group: $F_{2,43} = 2.8$, $p = 0.07$; group x shock: $F_{20,430} = 0.7$, $p = 0.78$). A week later, as expected, relative to no-trauma controls, trauma-exposed animals treated with vehicle exhibited associative fear in the trauma recall test (Figure 2C; $t_{25} = 10.5$, $p < 0.001$), increases in anxiety-related behavior in the light dark-test (Figure 2D; $t_{33.8} = 4.8$, $p < 0.001$), and heightened fear of the novel stressor environment (Figure 2E; $t_{29.7} = 4.1$, $p < 0.001$). Anisomycin administration immediately after trauma reduced all of these stress-induced defensive behaviors relative to trauma-exposed animals given vehicle (Figures 2C–2E; trauma recall: $t_{26.1} = 9.5$, $p < 0.001$; light-dark test: $t_{36.1} = 2.8$, $p < 0.01$; novel stressor: $t_{33.6} = 3.4$,

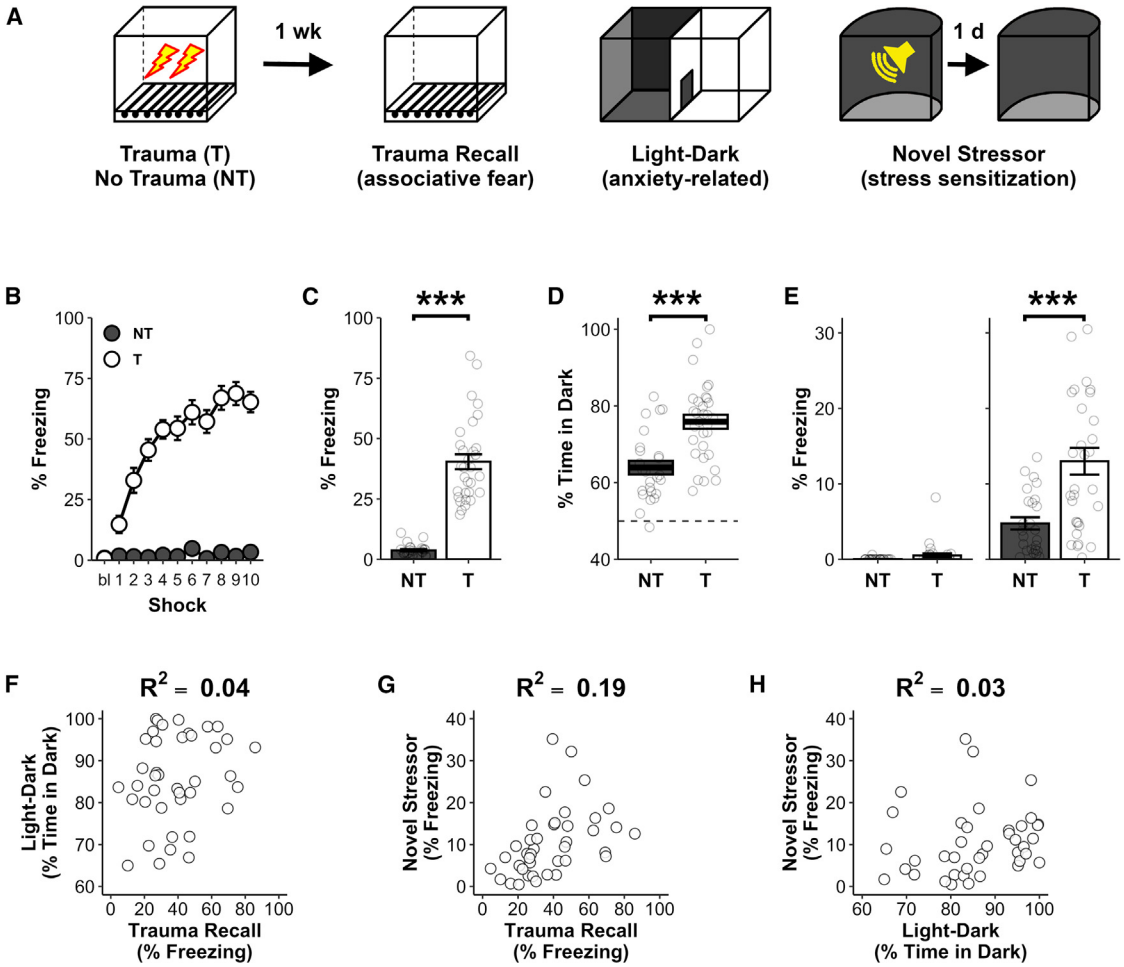


Figure 1. Acute stress produces multiple lasting changes in defensive behavior

(A) Animals were exposed to an environment in which they received 10 footshocks (trauma [T]) or were placed in the same environment and received no footshocks (no trauma [NT]). A week later, they were tested for associative fear of the trauma environment, anxiety-related behavior in the light-dark test, and their response to a novel stressor in a new environment to assess stress sensitization.

(B) Trauma-exposed animals displayed high levels of post-shock freezing during the trauma.

(C) Trauma-exposed animals displayed strong associative fear of the trauma environment.

(D) Trauma-exposed animals displayed increased anxiety-related behavior in the light-dark test.

(E) Trauma-exposed animals did not differ in baseline levels of freezing when initially placed in the environment of the novel stressor (left) but displayed increased fear of the novel stressor environment when returned to this environment the next day, evidence of stress sensitization (right).

(F) Correlation between trauma recall and anxiety-related behavior in light-dark test.

(G) Correlation between trauma recall and novel stressor response.

(H) Correlation between anxiety-related behavior in light-dark test and novel stressor response.

For (B)–(E), NT = 25 (13 female) and T = 31 (16 female) mice. For (F)–(H), T = 40 mice. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Error bars reflect standard error of the mean.

$p < 0.01$). However, anisomycin given 48 h after trauma did not reduce defensive behaviors relative to trauma-exposed animals given vehicle (Figures 2C–2E; trauma recall: $t_{13.3} = 0.1$, $p = 0.95$; light-dark test: $t_{24.7} = 0.2$, $p = 0.83$; novel stressor: $t_{12} = 0.7$, $p = 0.49$). Therefore, protein synthesis occurring just after trauma appears critical to the induction of the observed defensive phenotypes.

Next, we assessed the impacts of targeting trauma-induced protein synthesis specifically in the BLA or vHC, regions previously linked to regulating defensive behaviors and anxiety disorders (Figures 2F–2J).[70–73] Mice had indwelling cannulas implanted above either the BLA or vHC (Figure 2J; see Figure S3 for placement in all animals). After surgical recovery, animals then underwent trauma and immediately thereafter received intracranial infusions of anisomycin or vehicle. Alternatively, they experienced no trauma and were treated with vehicle. Animals treated with vehicle in the BLA and vHC showed no behavioral differences and are collapsed here into a single group (Figure S3). Prior to vehicle/anisomycin treatment, no differences were observed in freezing during the trauma session for animals

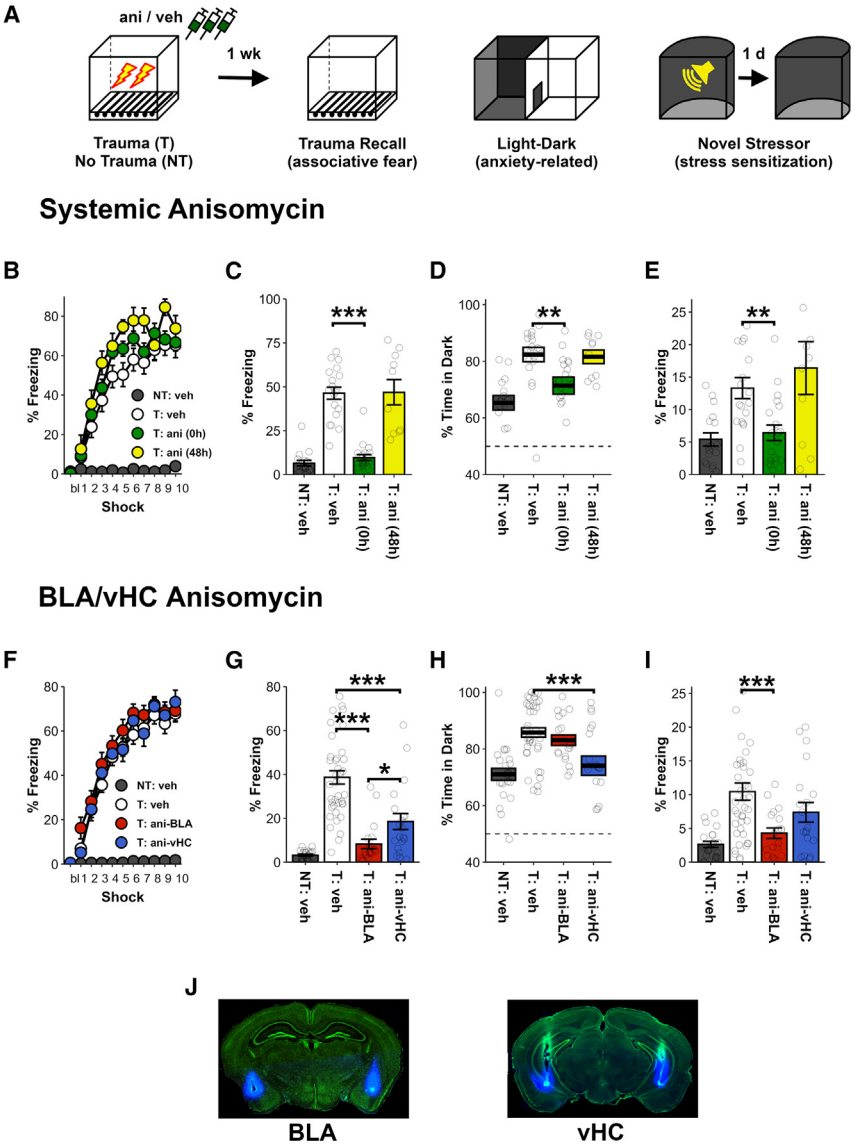


Figure 2. Stress-induced protein synthesis in the BLA and vHC supports distinct changes in defensive behavior

(A) After trauma (T) or no trauma (NT), animals were administered 3 injections of anisomycin (ani) or vehicle (veh). A week later, they were tested for associative fear of the trauma environment, anxiety-related behavior in the light-dark test, and their response to a novel stressor in a new environment.

(B) No differences were observed between trauma-exposed animals during the initial trauma.

(C) Anisomycin given systemically immediately after trauma, but not 48 h later, reduced associative fear of the trauma environment.

(D) Anisomycin given systemically immediately after trauma, but not 48 h later, reduced anxiety-related behavior in the light-dark test.

(E) Anisomycin given systemically immediately after trauma, but not 48 h later, reduced stress sensitization.

(F) No differences were observed between trauma-exposed animals during the initial trauma.

(G) Anisomycin in the BLA and vHC reduced associative fear of the trauma environment.

(H) Anisomycin in the vHC, but not the BLA, reduced anxiety-related behavior in the light-dark test.

(I) Anisomycin in the BLA, but not the vHC, reduced stress sensitization.

(J) Example placement of cannula injectors in the BLA and vHC for intracranial infusions.

For (B)–(E), anisomycin/vehicle was administered systemically either immediately (0h) or 48 h (48h) after trauma. For (F)–(J), anisomycin/vehicle was administered directly into either the BLA or vHC immediately after trauma. For systemic injections (B–E), NT: veh = 17 (5 female), T: veh = 19 (5 female), T: ani (0h) = 20 (5 female), and T: ani (0h) = 10 (5 female) mice. For intracranial infusions (F–J), NT: veh = 23, T: veh = 40, T: ani-BLA = 19, and T: ani-vHC = 20 mice. Half of the vehicle-treated animals had a cannula in the BLA and the other half in the vHC. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Error bars reflect standard error of the mean. Statistics are presented in the main text.

that underwent trauma (Figure 2F; group: $F_{2,76} = 1.1$, $p = 0.33$; group x shock: $F_{20,760} = 0.5$, $p = 0.96$). As anticipated, relative to no-trauma controls, trauma-exposed animals treated with vehicle exhibited a strong associative fear response (Figure 2G; $t_{40.7} = 11.5$, $p < 0.001$), heightened anxiety-related behavior in the light-dark test (Figure 2H; $t_{47.5} = 5.5$, $p < 0.001$), and heightened fear of the novel stressor (Figure 2I; $t_{47.6} = 5.8$, $p < 0.001$). These behaviors were differentially affected by blocking trauma-induced protein synthesis in the BLA and vHC. In the trauma recall test, anisomycin in either the BLA or vHC was effective at reducing associative freezing relative to trauma controls (Figure 2G; BLA: $t_{56.9} = 8.2$, $p < 0.001$; vHC: $t_{44} = 4.2$, $p < 0.001$), though anisomycin in the BLA did so to a greater extent (Figure 2G; BLA-ani vs. vHC-ani: $t_{30.5} = 2.4$, $p = 0.02$). In the light-dark test, anisomycin in the vHC greatly attenuated trauma-induced increases in anxiety-related behavior (Figure 2H; $t_{28.2} = 3.1$, $p < 0.01$). However, anisomycin in the BLA was without effect (Figure 2H; $t_{44.9} = 1.1$, $p = 0.28$). Lastly, anisomycin in the BLA was able to block the enhanced sensitivity to a novel stressor, whereas anisomycin in the vHC was without effect (Figure 2I; BLA: $t_{56.4} = 4.1$, $p < 0.001$; vHC: $t_{46.2} = 1.6$, $p = 0.12$). Notably, the dose of anisomycin used here was found to alter memory consolidation and protein synthesis but not memory expression, suggesting it did not acutely influence neuronal activity (Figure S4). Moreover, although the dosing regimen used was found to alter memory consolidation when given immediately after a learning event, it had no long-term deleterious impacts on future learning, indicating that no permanent damage was produced by anisomycin administration (Figure S4).

These findings highlight that while stress-induced protein synthesis in the BLA is paramount for associative fear and heightened stress sensitivity, it is not necessary for alterations in anxiety-related behavior. Conversely, stress-induced protein synthesis in the vHC is essential for increased anxiety-related

behavior, and to a lesser extent associative fear, but not heightened stress sensitivity. Importantly, the finding that blockade of protein synthesis in the BLA produced a profound impairment in associative fear of the trauma environment but no detectable effect on the light-dark test further suggests a dissociation between anxiety-related behavior and associative fear. In the same vein, the fact that blockade of protein synthesis in the vHC reduced associative fear but did not alter stress sensitization indicates that these phenotypes are also dissociable.

Neuronal activity in the BLA and vHC supports distinct stress-induced defensive behaviors

The prior findings indicate that stress-induced protein synthesis within the BLA and vHC supports the induction of distinct post-stress defensive phenotypes. However, neuronal activity in both regions could potentially still be necessary at a later time point to express changes in stress sensitivity and anxiety-related behavior. To evaluate this possibility, we next used a chemogenetic system[74] to inhibit either the BLA or vHC during testing of associative memory recall, anxiety-related behavior, and stress sensitization.

We first verified that administration of the agonist CNO-dihydrochloride (cno) was able to inhibit the neuronal activity of cells expressing the HM4D receptor in the BLA/vHC. A pan-neuronal virus expressing HM4D was infused into either the BLA or vHC (Figure 3A). Recording from these neurons in slices approximately 1 month later, we found robust inhibition of HM4D-expressing cells when cno was applied to the bath (Figure 3B; HM4D+ cells: pre-post: $F_{1,10} = 137$, $p < 0.001$, and pre-post x region: $F_{1,10} = 0.4$, $p = 0.52$; HM4D− cells: pre-post: $F_{1,1} = 0.2$, $p = 0.76$).

We next tested whether neuronal activity within the BLA and vHC is necessary for trauma memory recall (Figures 3C–3E). Animals expressing HM4D in either the BLA or vHC underwent trauma (Figure 3D), and a week later, their associative recall of the event was assessed—first during a drug-free, baseline test and then after receiving an injection of saline (veh) or cno (3 mg/kg, intraperitoneal [i.p.]). As expected, inhibition of either the BLA or vHC reduced freezing levels relative to baseline (Figure 3E; cno: $F_{1,14} = 33.5$, $p < 0.001$; cno x region: $F_{1,14} = 3.2$, $p = 0.1$), whereas vehicle-treated animals did not show altered freezing levels (Figure 3E; veh: $F_{1,12} = 2.8$, $p = 0.12$; veh x region: $F_{1,14} = 2.2$, $p = 0.16$). These findings mirror our protein synthesis results and are consistent with prior results showing that the activity of both the BLA and vHC is important for associative fear.[75]

Then, to assess the contributions of BLA and vHC neural activity to the expression of enhanced anxiety-related behavior and stress sensitivity after trauma, a virus expressing HM4D was infused into the BLA/vHC, or a control virus expressing EGFP was infused (Figure S5). Controls with EGFP in the BLA or vHC were collapsed into a common control group (EGFP: BLA/vHC; see Table S1 for comparisons of BLA/vHC controls). A month later, all animals underwent the trauma procedure (Figure 3F). Notably, no behavioral differences were observed during the initial trauma, suggesting that expression of the receptor alone had no effect on the acquisition or expression of conditioned fear (Figure 3G; group: $F_{2,65} = 0.2$, $p = 0.82$; group x shock: $F_{20,650} = 0.6$, $p = 0.84$). Additionally, when animals were placed in the trauma environment a week later, drug-free, no group differences in freezing were observed (Figure 3H; group: $F_{2,65} = 0.1$, $p = 0.88$). Consistent with our finding that stress-induced protein synthesis in the vHC supports enhancements in anxiety-related behavior, inhibition of the vHC produced a dramatic decrease in time spent on the dark side in the light-dark test (Figure 3I; $t_{29.2} = 3.3$, $p < 0.01$), whereas inhibition of the BLA was without effect (Figure 3I; $t_{45.8} = 0.4$, $p = 0.71$). Conversely, in the test of stress sensitization, animals in which the BLA was inhibited froze less than controls (Figure 3J; $t_{41.7} = 2.8$, $p < 0.01$), whereas inhibition of the vHC was without effect (Figure 3J; $t_{40.4} = 0.7$, $p = 0.52$).

To confirm the reliability of these findings, utilizing a different chemogenetic receptor,[76] we replicated the findings that BLA activity supports heightened stress sensitivity, whereas the vHC supports heightened anxiety-related behavior, and both of these structures support associative memory recall (Figure S6). Moreover, to test whether our finding that the vHC selectively contributes to anxiety-related behavior is generalizable, we tested the impacts of inhibiting the BLA and vHC across anxiety-related behavior tests (light-dark, elevated-plus maze, and open field), as each test is likely to tap into slightly different cognitive/behavioral processes. Although slight differences were observed across tests and chemogenetic systems, we broadly found that inhibiting the vHC, but not the BLA, was able to reduce anxiety-related behavior (Figure S7).

In summary, inactivation of the BLA/vHC mirrored the effects observed with protein synthesis inhibition, such that the BLA supports heightened stress sensitivity and the vHC supports enhanced anxiety-related behavior. Therefore, it appears that at the levels of both protein synthesis and neuronal activity, changes in stress sensitivity and anxiety-related behavior are supported by different neural substrates.

Inhibiting reciprocal BLA-vHC connections fails to alter stress sensitivity and anxiety-related behavior

Above, we have demonstrated that stress-induced protein synthesis and subsequent neuronal activity within the BLA and vHC support distinct defensive behavioral changes. This is incredibly surprising, given that the BLA and vHC share reciprocal monosynaptic connections,[77–79] and prior reports indicating these projections support at least some defensive behaviors in stress-naive animals.[79–81] That said, both the BLA and vHC contain output neurons that project to distinct downstream structures,[77,78,80–82] and there is evidence that these projections can contribute to different defensive processes.[80] Therefore, it may be that stress-induced changes in anxiety-related behavior and stress sensitivity are not dependent upon BLA-vHC connectivity. To test this possibility, we used a retrograde viral approach to selectively inhibit cells in the BLA that project to the vHC, or vice versa. A cre-expressing retrograde adeno-associated virus (AAV) was injected into either the BLA or vHC, and a cre-dependent HM4D virus or a control virus expressing only mCherry was injected into the other structure (Figure 4A; animals with mCherry in the BLA and vHC were collapsed into a single group; see Table S1 for comparisons of BLA/vHC controls). A month later, animals underwent the trauma protocol previously described, and BLA-vHC projections were inhibited during the light-dark

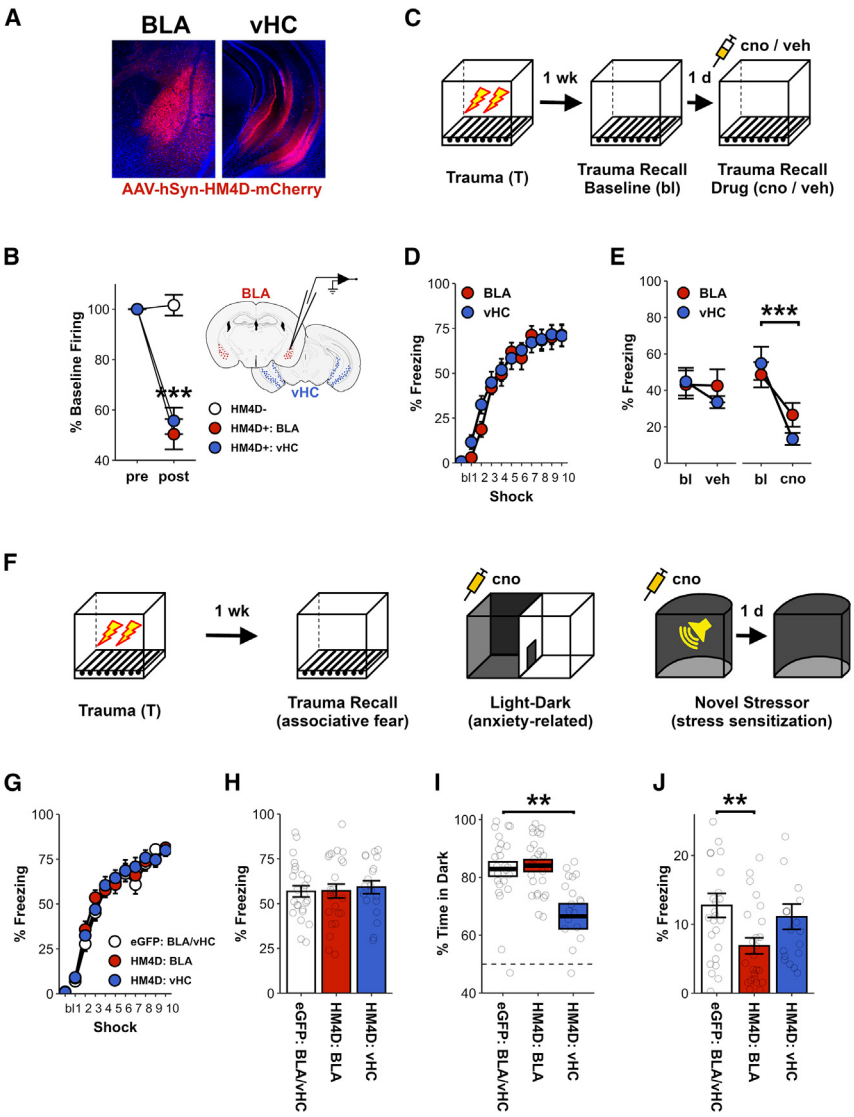


Figure 3. Neuronal activity in the BLA and vHC supports distinct stress-induced defensive behaviors

(A) A pan-neuronal virus expressing the inhibitory chemogenetic receptor HM4D, or EGFP, was infused in the BLA or vHC.

(B) HM4D+ neurons, as well as neighboring HM4D− neurons, were recorded before and after cno application. Application of cno dramatically reduced action potentials in HM4D+ neurons.

(C) Animals underwent trauma and a week later were tested twice for their associative recall of the traumatic event, first in a drug-free baseline test (bl), and second, after receiving an injection of cno or saline (veh).

(D) Animals with HM4D in the BLA and vHC did not differ during the initial trauma.

(E) Administration of cno reduced freezing in animals with HM4D in either the BLA or vHC.

(F) Animals underwent trauma, and a week later, they were tested for associative fear of the trauma environment, anxiety-related behavior in the light-dark test, and their response to a novel stressor in a new environment. The BLA/vHC were inhibited via cno administration prior to the light-dark test, as well as prior to the novel stressor.

(G) No group differences were observed during the initial trauma.

(H) No group differences were observed during the drug-free trauma recall test.

(I) Inhibition of the vHC, but not the BLA, reduced anxiety-related behavior in the light-dark test.

(J) Inhibition of the BLA, but not the vHC, reduced freezing in the test of stress sensitization.

For electrophysiological recordings in (B), HM4D− = 2, HM4D+: BLA = 6, and HM4D+: vHC = 7 cells. For effects of inhibition on recall in (C)–(E), BLA: veh = 7, BLA: cno = 7, vHC: veh = 7, and vHC: cno = 9 mice. For effects of inhibition on light-dark and novel stressor in (F)–(J), EGFP: BLA/vHC = 25, HM4D: BLA = 24, and HM4D: vHC = 19 mice. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Error bars reflect standard error of the mean. Statistics are presented in the main text.

test as well as during the novel stressor (Figure 4B). As expected, with both structures "online," the groups did not differ in their response to the initial trauma (Figure 4C; group: $F_{2,30} = 0.1$, $p = 0.91$; group x shock: $F_{20,300} = 0.7$, $p = 0.74$), nor did they differ in the trauma recall test (Figure 4D; group: $F_{2,30} = 1.9$, $p = 0.17$). What is striking is that selective inhibition of either projection did not alter anxiety-related behavior in the light-dark test (Figure 4E; group: $F_{2,30} = 1.1$, $p = 0.35$). Similarly, inhibiting either projection did not alter the response to the novel stressor in the test of stress sensitization (Figure 4F; group: $F_{2,30} = 2.2$, $p = 0.13$). Consequently, it appears that the reciprocal connections between the BLA and vHC do not play a pivotal role in these specific stress-induced changes in defensive behavior.

DISCUSSION

Associative learning frameworks—in which cues present at the time of a stressor come to drive behavior—have dominated how we study the impacts of stress on fear and anxiety disorders. Consequently, immense gains have been made in our understanding of the biological basis of associative fear learning, as well how these associations are extinguished. However, relatively little attention has been paid to non-associative learning processes governing stress-induced changes in defensive behavior. Here, utilizing a combination of targeted protein synthesis inhibition, chemogenetic inhibition, and projection-specific inhibition strategies, we demonstrate that the BLA and vHC differentially contribute to stress-induced changes in defensive behavior. Specifically, we find that although both structures contribute to associative learning and recall about a stressful event, they have dissociable contributions to non-associative learning processes: the BLA supports heightened sensitivity to subsequent aversive events, whereas the vHC supports increases in anxiety-related behavior. These findings highlight how associative and non-associative memories may be formed for a single stressful experience and suggest that the BLA and

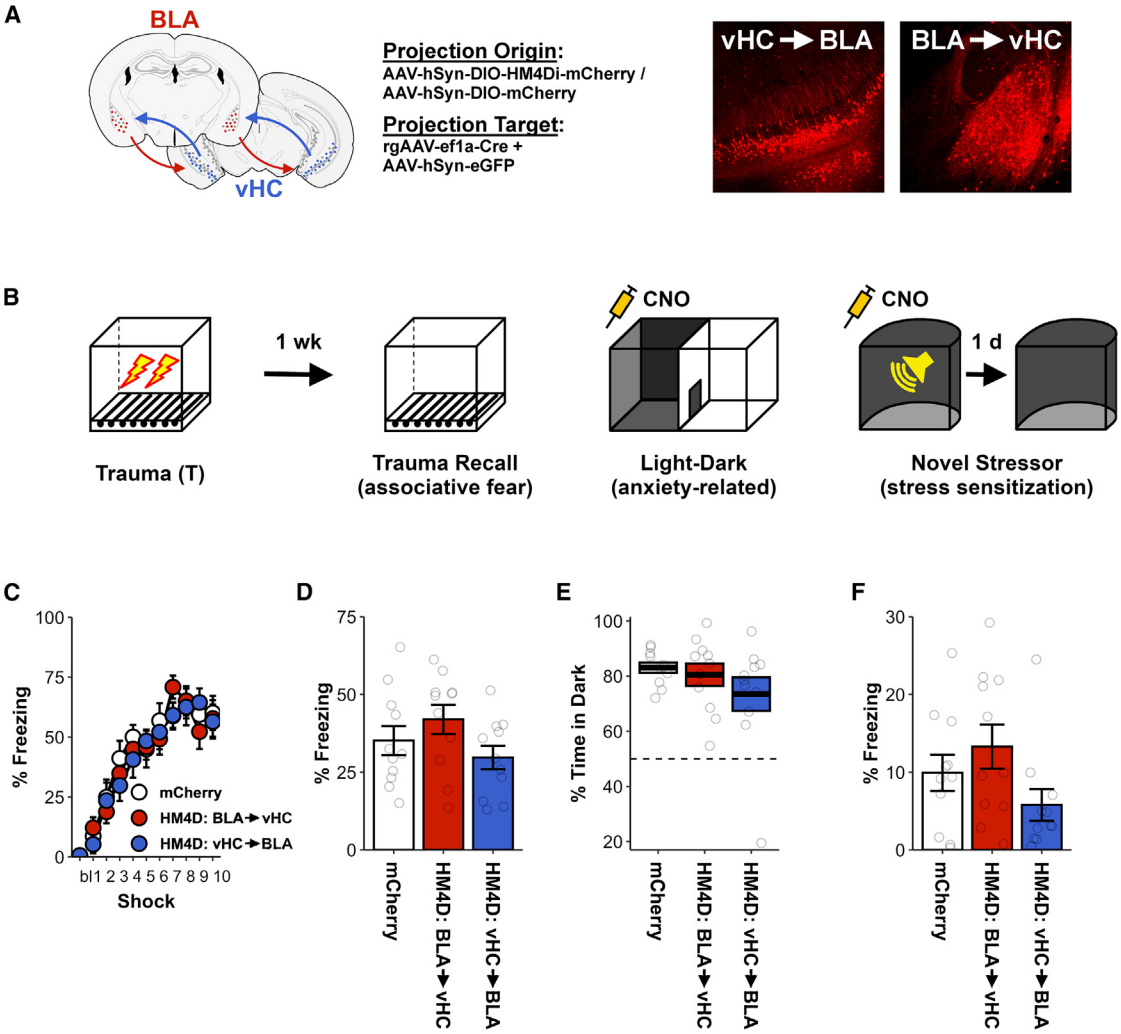


Figure 4. Reciprocal BLA-vHC connections do not support stress-induced changes in anxiety-related behavior or stress sensitivity

(A) Projection-specific targeting of BLA cells projecting to the vHC, or vice versa, was accomplished by infusing a cre-expressing retrograde virus into the projection target structure and cre-dependent HM4D/control virus into the projection origin structure. EGFP-expressing virus was co-infused into the projection target to confirm surgical placement.

(B) Animals underwent trauma and a week later were tested for associative fear of the trauma environment, anxiety-related behavior in the light-dark test, and their response to a novel stressor in a new environment. BLA-vHC connections were inhibited via cno administration prior to the light-dark test, as well as prior to the novel stressor.

(C) No group differences were observed during the initial trauma.

(D) No group differences were observed during the trauma recall test.

(E) No group differences were observed during the light-dark test of anxiety-related behavior.

(F) No group differences were observed during the novel stressor test for stress sensitization.

mCherry = 11, BLA→vHC = 11, and vHC→BLA = 11 mice. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Error bars reflect standard error of the mean. Statistics are presented in the main text.

vHC can participate in distinct brain networks to regulate defensive behaviors.

A wealth of literature supports the notion that the BLA and vHC regulate defensive behaviors.[43–54] In light of reciprocal connections between these structures,[77,83,84] it is often thought that stress-induced plasticity within the BLA and vHC, as well as their coordinated neuronal activity, subserves a common defensive process (or processes). While evidence exists that the connectivity of these structures plays an important role in defensive behavior,[79–81,85] our findings demonstrate that this is not always the case.

First, stress-induced protein synthesis within the BLA was found to be critical to subsequent enhancements in stress sensitivity, whereas stress-induced protein synthesis within the vHC had no bearing on this defensive phenotype. Conversely, stress-induced protein synthesis within the vHC, but not the BLA, was found to support heightened anxiety-related behaviors. Therefore, the fundamental structural plasticity that

supports these behavioral changes appears to emerge from distinct brain regions.

Second, it could be the case that neuronal activity within a brain region is necessary to express a particular behavioral change, even when plasticity in that region was not required for that behavioral change to come about. For instance, plasticity in an upstream region (e.g., the vHC) may result in subsequent neural activity changes in a downstream region (e.g., the BLA), which are, in turn, necessary for a given behavior (e.g., anxiety-related behavior). Ruling out this possibility, we found that suppressing neural activity within the BLA and vHC had doubly dissociable impacts on the expression of these behavioral changes. Inhibiting neural activity in the BLA was able to block the heightened response to aversive stimuli observed after an initial stressor, whereas inhibition of the vHC was without effect. Similarly, inhibiting neural activity in the vHC was able to block stress-induced changes in anxiety-related behavior, while inhibition of the BLA was without effect. Accordingly, at the levels of both plasticity and neuronal activity, the BLA and vHC appear to differentially regulate these behaviors.

The above findings are surprising, given the reciprocal monosynaptic connections between the BLA and vHC. In light of this, we attempted to replicate the effects of BLA/vHC inhibition, this time targeting only neurons in the BLA that project to the vHC or only neurons in the vHC that project to the BLA. Consistent with the hypothesis that the BLA and vHC regulate these behaviors independently, inhibition of either projection was without effect. Therefore, it seems that alternative projections emanating from the BLA and vHC support these behavioral changes. In the future, we hope to identify these targets, as well as the broader brain networks supporting changes in stress sensitivity and anxiety-related behavior.

Regarding the lack of effect of inhibiting BLA-vHC connections, it could be that polysynaptic connectivity between the BLA and vHC might instead support changes in stress sensitivity and anxiety-related behavior. Careful consideration of our experiments targeting activity in the BLA and vHC with a pan-neuronal promoter suggests otherwise. If polysynaptic connectivity between the BLA and vHC were critical to stress sensitivity and anxiety-related behavior, then inhibition of either structure would be expected to disrupt both behaviors. However, this was not the case.

The dissociation of the contributions of the BLA and vHC to the defensive behaviors studied here is not entirely without precedent, although side-by-side comparisons of their functions are limited. For instance, despite the large amount of literature on the role of the BLA in associative fear learning,[9–11,43,44,86–89] several studies have reported that inhibition of the BLA is without effect on exploratory anxiety-related behaviors,[79,90–92] although discrepancies also exist.[93] Additionally, a recent report found that optogenetic stimulation of projections from the vHC to the BLA do not regulate anxiety-related behavior.[80] This corroborates the hypothesis that the vHC regulates exploratory anxiety-related behavior through its connections with other downstream structures, such as the hypothalamus[80,94,95] or medial prefrontal cortex.[46] While stimulation of BLA terminal fibers in the vHC has been found to alter anxiety-related behavior,[79] this may reflect a general effect of exciting the vHC as opposed to the natural role served by BLA to vHC efferents. Indeed, we were unable to alter anxiety-related behavior via chemogenetic inhibition of vHC-projecting BLA neurons. Together, these results broadly suggest that the vHC may regulate exploratory anxiety-related behavior in a manner distinct from its connections with the BLA.

Our findings also add to existing evidence that associative and non-associative impacts of stress are biologically distinct. Blockade of stress-induced protein synthesis in both the BLA and vHC was able to impair the acquisition of associative fear for the context of an initial stressor, and blockade of activity within either structure was similarly able to impair associative memory recall. Importantly, if the observed changes in anxiety-related behavior and stress sensitivity were dependent upon the associative memory of the stressor, then blocking associative memory for the stressor should impair their expression. However, manipulations of the BLA potently reduced associative fear but did not affect increases in anxiety-related behavior. Similarly, manipulations of the vHC reduced associative fear but did not interfere with stress sensitization. Additionally, it does not seem that these discrepancies are a matter of thresholding (e.g., manipulations of the BLA did not alter the associative memory enough to alter anxiety-related behavior). Blockade of stress-induced protein synthesis in the BLA was more effective at reducing subsequent associative fear of the stressor context than blockade of stress-induced protein synthesis in the vHC. Nevertheless, blocking protein synthesis in the vHC reduced subsequent anxiety-related behavior, whereas the same manipulation in the BLA did not. Therefore, it seems that each of these defensive phenotypes—associative memory, heightened stress sensitivity, and anxiety-related behavior—reflect distinct plasticity mechanisms.

Protein synthesis within the BLA was necessary for the acquisition and expression of both heightened associative memories of a stressful event and the heightened sensitivity to novel stressors after prior stress. Furthermore, these two phenotypes were correlated, albeit weakly. It may be argued that these defensive phenotypes are one in the same. Prior evidence, as well as data presented here, stands in opposition to this possibility. First, extinction of the associative memory for an initial stressor has been found to leave the enhanced response to a second stressor intact.[15,38,39] Second, early-life stress at a time point when rodents are not able to form associative memories nevertheless leaves animals with heightened responses to subsequent stressors in adulthood.[14] Third, pharmacological blockade of NMDA receptors during an initial stressor, which produces near-complete loss of the associative memory for that event, does not reduce heightened responding to subsequent aversive events.[15] Lastly, here we show that inactivation of the BLA and vHC was able to equivalently impair trauma memory recall, but only inactivation of the BLA was able to alter the heightened sensitivity to a novel stressor. Therefore, despite both phenotypes' dependence upon the BLA, associative memory for a stressor and the enhanced responding to subsequent stress are dissociable. It could be that synapse- and ensemble-specific plasticity within the amygdala supports the associative memory for a specific stressor, whereas a broader form of non-associative plasticity within the amygdala supports

sensitized stress responses. Future studies will disentangle how plasticity within the amygdala supports these two forms of learning.

In closing, these results shed light on how stress-induced plasticity within the BLA and vHC supports the formation of defensive behavioral phenotypes relevant to neuropsychiatric illness. Furthermore, they highlight just how distinct memories of stressful events might be. Similar to the separate memory systems in the brain supporting episodic and procedural learning of the same event, we find that different defensive behaviors induced by stress are supported by distinct brain regions. This has important clinical implications for the treatment of anxiety disorders and other stress-associated mental health conditions. First, it suggests that clinically targeting one stress-induced defensive behavior, or the circuits that support that behavior, may leave others wholly unaffected. Perhaps some of the existing gaps in treatment result from a failure to adequately target the spectrum of defensive processes altered in these conditions. Second, by understanding the relationship between specific defensive behaviors and their biology, we may make greater headway in the treatment of these conditions. For instance, associative learning processes are more likely affected in some mental health conditions (e.g., post-traumatic stress disorder [PTSD]), while anxiety-related behaviors might be more affected in others (e.g., generalized anxiety disorder), and these differences undoubtedly covary with different neuronal patterns. Indeed, it is known that different anxiety disorders are not only symptomatically different but are characterized by unique brain activity patterns.[71] By understanding these inter-relationships, we may more rapidly find the appropriate key to unlock the door to recovery for stress-associated mental health conditions.

Limitations of the study

We have examined one of the most commonly utilized stressors (contextual fear conditioning with high-intensity footshock) and its lasting impacts on subsequent defensive behaviors. This stressor was utilized because, unlike other commonly utilized acute stressors (e.g., restraint, forced swim), it provides a robust and reliable metric of associative fear that can be parametrically manipulated. This gave us the ability to systematically study the circuit mechanisms supporting associative vs. non-associative impacts of stress. Additionally, the brief nature of this stressor allowed us to readily intervene in the memory consolidation process (as opposed to chronic stressors that have a more protracted consolidation). Although future studies will be necessary to determine how our results generalize to other forms of stress, we hope these findings elevate the idea that the consequences of stress can be supported by divergent plasticity processes, as opposed to a monolith.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact, Denise J. Cai (denisecai@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data and statistical analysis are available at https://github.com/ZachPenn/2024_CellReports and DOI: https://zenodo.org/records/13371993.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

This work was supported by NIMH DP2 MH122399, R01 MH120162, R56 MH132959, the Brain Research Foundation Award, the Klingenstein-Simons Fellowship, the Brain & Behavior Research Foundation Young Investigator Award, the McKnight Memory and Cognitive Disorders Award, the One Mind Rising Star Award, the Hirschl/Weill-Caulier Research Award, the McKnight Brain Research Foundation & American Foundation for Aging Research Innovator Award in Cognitive Aging and Memory Loss, the Chan Zuckerberg Initiative Friedman Brain Institute Research Scholars Award, and the Mount Sinai Distinguished Scholar Award to D.J.C.; NIMH K99 MH131792, the Brain & Behavior Research Foundation Young Investigator Award, and the Mount Sinai Friedman Brain Institute Postdoc Innovator Award to Z.T.P.; NINDS F32 NS116416 to Z.C.W.; the AES Predoctoral Research Fellowship to Y.F.; funds from the Howard Hughes Medical Institute to I.M.; and NINDS R01 NS116357, NIA RF1 AG072497, and NINDS R01 NS136590 to T.S. We thank Kathleen Wang for helping to create the graphical abstract for this article. The authors would like to thank Dr. Scott Russo and Dr. Roger Clem for their helpful comments on this work.

AUTHOR CONTRIBUTIONS

Z.T.P. and D.J.C. conceived of the overarching research goals, designed the experiments, and oversaw the experiments. Z.T.P. analyzed the experimental data and prepared the initial manuscript. Z.T.P., A.R.L., P.S., S.D.A.-R., B.K., Z.C.W., Y.F., Z.D., M.E.B., T.R.F., L.C., S.L.F., I.M., T.S., and D.J.C. contributed to the interpretation of the results and edited the manuscript. Z.T.P., A.L.B., P.S., S.D.A.-R., B.K., Z.C.W., Y.F., Z.D., M.E.B., T.R.F., L.C., and S.L.F. performed experiments. Z.T.P. and Z.D. designed the software for the analysis of behavioral data. D.J.C., I.M., T.S., Z.T.P., Z.C.W., and Y.F. secured funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - Behavioral testing
  - Surgery
  - Anisomycin experiments
  - HM4D experiments
  - PSAM experiments
  - Histology
  - *Ex vivo* electrophysiology
  - Immunohistochemistry and image analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2024.114871.

Received: March 23, 2023
Revised: July 1, 2024
Accepted: September 27, 2024

REFERENCES

1. Fendt, M., and Fanselow, M.S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. Neurosci. Biobehav. Rev. *23*, 743–760.
2. Bolles, R.C. (1970). Species-specific defense reations and avoidance learning. Psychol. Rev. *77*, 32–48.
3. Fanselow, M.S., and Lester, L.S. (1988). A functional behavioristic approach to aversively motivated behavior. In Evolution and Learning, R.C. Bolles and M.C. Beecher, eds. (Erlbaum), pp. 185–211.
4. Cannon, W.B. (1915). Bodily Changes in Pain, Hunger, Fear and Rage: An Account of Recent Researches into the Function of Emotional Excitement (D. Appleton and Componay).
5. Blanchard, R.J., Blanchard, D.C., Rodgers, J., and Weiss, S.M. (1990). The characterization and modelling of antipredator defensive behavior. Neurosci. Biobehav. Rev. *14*, 463–472. https://doi.org/10.1016/s0149-7634(05)80069-7.
6. Blanchard, R.J., and Blanchard, D.C. (1969). Passive and active reactions to fear-eliciting stimuli. J. Comp. Physiol. Psychol. *68*, 129–135. https://doi.org/10.1037/h0027676.
7. Bienvenu, T.C.M., Dejean, C., Jercog, D., Aouizerate, B., Lemoine, M., and Herry, C. (2021). The advent of fear conditioning as an animal model of post-traumatic stress disorder: Learning from the past to shape the future of PTSD research. Neuron *109*, 2380–2397. https://doi.org/10.1016/j.neuron.2021.05.017.
8. Maren, S. (2005). Synaptic mechanisms of associative memory in the amygdala. Neuron *47*, 783–786. https://doi.org/10.1016/j.neuron.2005.08.009.
9. Maren, S. (2003). The amygdala, synaptic plasticity, and fear memory. Ann. N. Y. Acad. Sci. *985*, 106–113.
10. Fanselow, M.S., and LeDoux, J.E. (1999). Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala. Neuron *23*, 229–232.
11. Davis, M. (1992). The role of the amygdala in fear and anxiety. Annu. Rev. Neurosci. *15*, 353–375. https://doi.org/10.1146/annurev.ne.15.030192.002033.
12. Fadok, J.P., Krabbe, S., Markovic, M., Courtin, J., Xu, C., Massi, L., Botta, P., Bylund, K., Müller, C., Kovacevic, A., et al. (2017). A competitive inhibitory circuit for selection of active and passive fear responses. Nature *542*, 96–100. https://doi.org/10.1038/nature21047.
13. Adamec, R.E., and Shallow, T. (1993). Lasting effects on rodent anxiety of a single exposure to a cat. Physiol. Behav. *54*, 101–109. https://doi.org/10.1016/0031-9384(93)90050-.
14. Poulos, A.M., Reger, M., Mehta, N., Zhuravka, I., Sterlace, S.S., Gannam, C., Hovda, D.A., Giza, C.C., and Fanselow, M.S. (2014). Amnesia for early life stress does not preclude the adult development of posttraumatic stress disorder symptoms in rats. Biol. Psychiatr. *76*, 306–314. https://doi.org/10.1016/j.biopsych.2013.10.007.
15. Rau, V., DeCola, J.P., and Fanselow, M.S. (2005). Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. Neurosci. Biobehav. Rev. *29*, 1207–1223. https://doi.org/10.1016/j.neubiorev.2005.04.010.
16. Pynoos, R.S., Ritzmann, R.F., Steinberg, A.M., Goenjian, A., and Prisecaru, I. (1996). A behavioral animal model of posttraumatic stress disorder featuring repeated exposure to situational reminders. Biol. Psychiatr. *39*, 129–134. https://doi.org/10.1016/0006-3223(95)00088-7.
17. Servatius, R.J., Ottenweller, J.E., and Natelson, B.H. (1995). Delayed startle sensitization distinguishes rats exposed to one or three stress sessions: further evidence toward an animal model of PTSD. Biol. Psychiatr. *38*, 539–546. https://doi.org/10.1016/0006-3223(94)00369-E.
18. American Psychiatric Association (2013). American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (American Psychiatric Publishing).
19. Moreno-Peral, P., Conejo-Cerón, S., Motrico, E., Rodríguez-Morejón, A., Fernández, A., García-Campayo, J., Roca, M., Serrano-Blanco, A., Rubio-Valera, M., and Bellón, J.Á. (2014). Risk factors for the onset of panic and generalised anxiety disorders in the general adult population: a systematic review of cohort studies. J. Affect. Disord. *168*, 337–348. https://doi.org/10.1016/j.jad.2014.06.021.
20. Kessler, R.C., Gruber, M., Hettema, J.M., Hwang, I., Sampson, N., and Yonkers, K.A. (2008). Co-morbid major depression and generalized anxiety disorders in the National Comorbidity Survey follow-up. Psychol. Med. *38*, 365–374. https://doi.org/10.1017/S0033291707002012.
21. Blanco, C., Rubio, J., Wall, M., Wang, S., Jiu, C.J., and Kendler, K.S. (2014). Risk factors for anxiety disorders: common and specific effects in a national sample. Depress. Anxiety *31*, 756–764. https://doi.org/10.1002/da.22247.
22. Keiser, A.A., Turnbull, L.M., Darian, M.A., Feldman, D.E., Song, I., and Tronson, N.C. (2017). Sex Differences in Context Fear Generalization and Recruitment of Hippocampus and Amygdala during Retrieval. Neuropsychopharmacology *42*, 397–407. https://doi.org/10.1038/npp.2016.174.
23. Lissek, S., Bradford, D.E., Alvarez, R.P., Burton, P., Espensen-Sturges, T., Reynolds, R.C., and Grillon, C. (2014). Neural substrates of classically conditioned fear-generalization in humans: a parametric fMRI study. Soc. Cognit. Affect Neurosci. *9*, 1134–1142. https://doi.org/10.1093/scan/nst096.
24. Lissek, S., Kaczkurkin, A.N., Rabin, S., Geraci, M., Pine, D.S., and Grillon, C. (2014). Generalized anxiety disorder is associated with overgeneralization of classically conditioned fear. Biol. Psychiatr. *75*, 909–915. https://doi.org/10.1016/j.biopsych.2013.07.025.
25. Zelikowsky, M., Bissiere, S., Hast, T.A., Bennett, R.Z., Abdipranoto, A., Vissel, B., and Fanselow, M.S. (2013). Prefrontal microcircuit underlies contextual learning after hippocampal loss. Proc. Natl. Acad. Sci. USA *110*, 9938–9943. https://doi.org/10.1073/pnas.1301691110.
26. Xu, W., and Südhof, T.C. (2013). A neural circuit for memory specificity and generalization. Science *339*, 1290–1295. https://doi.org/10.1126/science.1229534.
27. Wiltgen, B.J., Zhou, M., Cai, Y., Balaji, J., Karlsson, M.G., Parivash, S.N., Li, W., and Silva, A.J. (2010). The hippocampus plays a selective role in the retrieval of detailed contextual memories. Curr. Biol. *20*, 1336–1344. https://doi.org/10.1016/j.cub.2010.06.068.
28. Starita, F., Kroes, M.C.W., Davachi, L., Phelps, E.A., and Dunsmoor, J.E. (2019). Threat learning promotes generalization of episodic memory. J. Exp. Psychol. Gen. *148*, 1426–1434. https://doi.org/10.1037/xge0000551.
29. Dunsmoor, J.E., Kroes, M.C.W., Braren, S.H., and Phelps, E.A. (2017). Threat intensity widens fear generalization gradients. Behav. Neurosci. *131*, 168–175. https://doi.org/10.1037/bne0000186.
30. Dunsmoor, J.E., Otto, A.R., and Phelps, E.A. (2017). Stress promotes generalization of older but not recent threat memories. Proc. Natl. Acad. Sci. USA *114*, 9218–9223. https://doi.org/10.1073/pnas.1704428114.
31. Davis, M. (1989). Neural systems involved in fear-potentiated startle. Ann. N. Y. Acad. Sci. *563*, 165–183. https://doi.org/10.1111/j.1749-6632.1989.tb42197.x.
32. Brown, J.S., Kalish, H.I., and Farber, I.E. (1951). Conditioned fear as revealed by magnitude of startle response to an auditory stimulus. J. Exp. Psychol. *41*, 317–328. https://doi.org/10.1037/h0060166.
33. Norrholm, S.D., Jovanovic, T., Olin, I.W., Sands, L.A., Karapanou, I., Bradley, B., and Ressler, K.J. (2011). Fear extinction in traumatized

civilians with posttraumatic stress disorder: relation to symptom severity. Biol. Psychiatr. *69*, 556–563. https://doi.org/10.1016/j.biopsych.2010.09.013.

34. Jovanovic, T., Blanding, N.Q., Norrholm, S.D., Duncan, E., Bradley, B., and Ressler, K.J. (2009). Childhood abuse is associated with increased startle reactivity in adulthood. Depress. Anxiety *26*, 1018–1026. https://doi.org/10.1002/da.20599.

35. Kaczkurkin, A.N., Burton, P.C., Chazin, S.M., Manbeck, A.B., Espensen-Sturges, T., Cooper, S.E., Sponheim, S.R., and Lissek, S. (2017). Neural Substrates of Overgeneralized Conditioned Fear in PTSD. Am. J. Psychiatr. *174*, 125–134. https://doi.org/10.1176/appi.ajp.2016.15121549.

36. Grillon, C., and Morgan, C.A. (1999). Fear-potentiated startle conditioning to explicit and contextual cues in Gulf War veterans with posttraumatic stress disorder. J. Abnorm. Psychol. *108*, 134–142.

37. Acheson, D.T., Geyer, M.A., Baker, D.G., Nievergelt, C.M., Yurgil, K., and Risbrough, V.B.; MRS-II Team (2015). Conditioned fear and extinction learning performance and its association with psychiatric symptoms in active duty Marines. Psychoneuroendocrinology *51*, 495–505. https://doi.org/10.1016/j.psyneuen.2014.09.030.

38. Long, V.A., and Fanselow, M.S. (2012). Stress-enhanced fear learning in rats is resistant to the effects of immediate massed extinction. Stress *15*, 627–636. https://doi.org/10.3109/10253890.2011.650251.

39. Hassien, A.M., Shue, F., Bernier, B.E., and Drew, M.R. (2020). A mouse model of stress-enhanced fear learning demonstrates extinction-sensitive and extinction-resistant effects of footshock stress. Behav. Brain Res. *379*, 112391. https://doi.org/10.1016/j.bbr.2019.112391.

40. Difede, J., Olden, M., and Cukor, J. (2014). Evidence-based treatment of post-traumatic stress disorder. Annu. Rev. Med. *65*, 319–332. https://doi.org/10.1146/annurev-med-051812-145438.

41. Bradley, R., Greene, J., Russ, E., Dutra, L., and Westen, D. (2005). A multidimensional meta-analysis of psychotherapy for PTSD. Am. J. Psychiatr. *162*, 214–227. https://doi.org/10.1176/appi.ajp.162.2.214.

42. Cukor, J., Olden, M., Lee, F., and Difede, J. (2010). Evidence-based treatments for PTSD, new directions, and special challenges. Ann. N. Y. Acad. Sci. *1208*, 82–89. https://doi.org/10.1111/j.1749-6632.2010.05793.x.

43. Maren, S., Aharonov, G., and Fanselow, M.S. (1996). Retrograde abolition of conditional fear after excitotoxic lesions in the basolateral amygdala of rats: absence of a temporal gradient. Behav. Neurosci. *110*, 718–726.

44. Blanchard, D.C., and Blanchard, R.J. (1972). Innate and conditioned reactions to threat in rats with amygdaloid lesions. J. Comp. Physiol. Psychol. *81*, 281–290.

45. Feinstein, J.S., Adolphs, R., Damasio, A., and Tranel, D. (2011). The human amygdala and the induction and experience of fear. Curr. Biol. *21*, 34–38. https://doi.org/10.1016/j.cub.2010.11.042.

46. Padilla-Coreano, N., Bolkan, S.S., Pierce, G.M., Blackman, D.R., Hardin, W.D., Garcia-Garcia, A.L., Spellman, T.J., and Gordon, J.A. (2016). Direct Ventral Hippocampal-Prefrontal Input Is Required for Anxiety-Related Neural Activity and Behavior. Neuron *89*, 857–866. https://doi.org/10.1016/j.neuron.2016.01.011.

47. Kheirbek, M.A., Drew, L.J., Burghardt, N.S., Costantini, D.O., Tannenholz, L., Ahmari, S.E., Zeng, H., Fenton, A.A., and Hen, R. (2013). Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus. Neuron *77*, 955–968. https://doi.org/10.1016/j.neuron.2012.12.038.

48. Adhikari, A., Topiwala, M.A., and Gordon, J.A. (2010). Synchronized activity between the ventral hippocampus and the medial prefrontal cortex during anxiety. Neuron *65*, 257–269. https://doi.org/10.1016/j.neuron.2009.12.002.

49. Fanselow, M.S., and Dong, H.W. (2010). Are the dorsal and ventral hippocampus functionally distinct structures? Neuron *65*, 7–19. https://doi.org/10.1016/j.neuron.2009.11.031.

50. Bannerman, D.M., Yee, B.K., Good, M.A., Heupel, M.J., Iversen, S.D., and Rawlins, J.N. (1999). Double dissociation of function within the hippocampus: a comparison of dorsal, ventral, and complete hippocampal cytotoxic lesions. Behav. Neurosci. *113*, 1170–1188. https://doi.org/10.1037//0735-7044.113.6.1170.

51. Han, J.H., Kushner, S.A., Yiu, A.P., Cole, C.J., Matynia, A., Brown, R.A., Neve, R.L., Guzowski, J.F., Silva, A.J., and Josselyn, S.A. (2007). Neuronal competition and selection during memory formation. Science *316*, 457–460. https://doi.org/10.1126/science.1139438.

52. Mastrodonato, A., Martinez, R., Pavlova, I.P., LaGamma, C.T., Brachman, R.A., Robison, A.J., and Denny, C.A. (2018). Ventral CA3 Activation Mediates Prophylactic Ketamine Efficacy Against Stress-Induced Depressive-like Behavior. Biol. Psychiatr. *84*, 846–856. https://doi.org/10.1016/j.biopsych.2018.02.011.

53. Zaki, Y., Mau, W., Cincotta, C., Monasterio, A., Odom, E., Doucette, E., Grella, S.L., Merfeld, E., Shpokayte, M., and Ramirez, S. (2022). Hippocampus and amygdala fear memory engrams re-emerge after contextual fear relapse. Neuropsychopharmacology *47*, 1992–2001. https://doi.org/10.1038/s41386-022-01407-0.

54. Ramirez, S., Liu, X., MacDonald, C.J., Moffa, A., Zhou, J., Redondo, R.L., and Tonegawa, S. (2015). Activating positive memory engrams suppresses depression-like behaviour. Nature *522*, 335–339. https://doi.org/10.1038/nature14514.

55. Stevens, J.S., Almli, L.M., Fani, N., Gutman, D.A., Bradley, B., Norrholm, S.D., Reiser, E., Ely, T.D., Dhanani, R., Glover, E.M., et al. (2014). PACAP receptor gene polymorphism impacts fear responses in the amygdala and hippocampus. Proc. Natl. Acad. Sci. USA *111*, 3158–3163. https://doi.org/10.1073/pnas.1318954111.

56. Perusini, J., Meyer, E., Rau, V., Avershal, J., Rajbhandari, A., Hoffman, A., Nocera, N., Condro, M., Waschek, J., Spigelman, I., and Fanselow, M. (2014). Mechanisms underlying the induction and expression of fear sensitization following acute traumatic stress (Pavlovian Society).

57. Rau, V., and Fanselow, M.S. (2009). Exposure to a stressor produces a long lasting enhancement of fear learning in rats. Stress *12*, 125–133. https://doi.org/10.1080/10253890802137320.

58. Pennington, Z.T., Trott, J.M., Rajbhandari, A.K., Li, K., Walwyn, W.M., Evans, C.J., and Fanselow, M.S. (2020). Chronic opioid pretreatment potentiates the sensitization of fear learning by trauma. Neuropsychopharmacology *45*, 482–490. https://doi.org/10.1038/s41386-019-0559-5.

59. Chaouloff, F., Durand, M., and Mormède, P. (1997). Anxiety- and activity-related effects of diazepam and chlordiazepoxide in the rat light/dark and dark/light tests. Behav. Brain Res. *85*, 27–35. https://doi.org/10.1016/s0166-4328(96)00160-x.

60. Crawley, J.N. (1981). Neuropharmacologic specificity of a simple animal model for the behavioral actions of benzodiazepines. Pharmacol. Biochem. Behav. *15*, 695–699. https://doi.org/10.1016/0091-3057(81)90007-1.

61. Kessler, R.C., Sonnega, A., Bromet, E., Hughes, M., and Nelson, C.B. (1995). Posttraumatic stress disorder in the National Comorbidity Survey. Arch. Gen. Psychiatr. *52*, 1048–1060.

62. Breslau, N., Davis, G.C., Andreski, P., Peterson, E.L., and Schultz, L.R. (1997). Sex differences in posttraumatic stress disorder. Arch. Gen. Psychiatr. *54*, 1044–1048.

63. Smith, A.C.W., Jonkman, S., Difeliceantonio, A.G., O'Connor, R.M., Ghoshal, S., Romano, M.F., Everitt, B.J., and Kenny, P.J. (2021). Opposing roles for striatonigral and striatopallidal neurons in dorsolateral striatum in consolidating new instrumental actions. Nat. Commun. *12*, 5121. https://doi.org/10.1038/s41467-021-25460-3.

64. Hernandez, P.J., Sadeghian, K., and Kelley, A.E. (2002). Early consolidation of instrumental learning requires protein synthesis in the nucleus accumbens. Nat. Neurosci. *5*, 1327–1331. https://doi.org/10.1038/nn973.

65. Santini, E., Ge, H., Ren, K., Peña de Ortiz, S., and Quirk, G.J. (2004). Consolidation of fear extinction requires protein synthesis in the medial

prefrontal cortex. J. Neurosci. *24*, 5704–5710. https://doi.org/10.1523/JNEUROSCI.0786-04.2004.

66. Schafe, G.E., and LeDoux, J.E. (2000). Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. J. Neurosci. *20*, RC96.
67. Nader, K., Schafe, G.E., and Le Doux, J.E. (2000). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. Nature *406*, 722–726. https://doi.org/10.1038/35021052.
68. Bourtchouladze, R., Abel, T., Berman, N., Gordon, R., Lapidus, K., and Kandel, E.R. (1998). Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. Learn. Mem. *5*, 365–374.
69. Kandel, E.R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. Science *294*, 1030–1038. https://doi.org/10.1126/science.1067020.
70. Gilbertson, M.W., Shenton, M.E., Ciszewski, A., Kasai, K., Lasko, N.B., Orr, S.P., and Pitman, R.K. (2002). Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma. Nat. Neurosci. *5*, 1242–1247. https://doi.org/10.1038/nn958.
71. Etkin, A., and Wager, T.D. (2007). Functional neuroimaging of anxiety: a meta-analysis of emotional processing in PTSD, social anxiety disorder, and specific phobia. Am. J. Psychiatr. *164*, 1476–1488. https://doi.org/10.1176/appi.ajp.2007.07030504.
72. Kühn, S., and Gallinat, J. (2013). Gray matter correlates of posttraumatic stress disorder: a quantitative meta-analysis. Biol. Psychiatr. *73*, 70–74. https://doi.org/10.1016/j.biopsych.2012.06.029.
73. Rabinak, C.A., Angstadt, M., Welsh, R.C., Kenndy, A.E., Lyubkin, M., Martis, B., and Phan, K.L. (2011). Altered amygdala resting-state functional connectivity in post-traumatic stress disorder. Front. Psychiatr. *2*, 62. https://doi.org/10.3389/fpsyt.2011.00062.
74. Armbruster, B.N., Li, X., Pausch, M.H., Herlitze, S., and Roth, B.L. (2007). Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. Proc. Natl. Acad. Sci. USA *104*, 5163–5168. https://doi.org/10.1073/pnas.0700293104.
75. Sierra-Mercado, D., Padilla-Coreano, N., and Quirk, G.J. (2011). Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. Neuropsychopharmacology *36*, 529–538. https://doi.org/10.1038/npp.2010.184.
76. Magnus, C.J., Lee, P.H., Bonaventura, J., Zemla, R., Gomez, J.L., Ramirez, M.H., Hu, X., Galvan, A., Basu, J., Michaelides, M., and Sternson, S.M. (2019). Ultrapotent chemogenetics for research and potential clinical applications. Science *364*, eaav5282. https://doi.org/10.1126/science.aav5282.
77. Hintiryan, H., Bowman, I., Johnson, D.L., Korobkova, L., Zhu, M., Khanjani, N., Gou, L., Gao, L., Yamashita, S., Bienkowski, M.S., et al. (2021). Connectivity characterization of the mouse basolateral amygdalar complex. Nat. Commun. *12*, 2859. https://doi.org/10.1038/s41467-021-22915-5.
78. Gergues, M.M., Han, K.J., Choi, H.S., Brown, B., Clausing, K.J., Turner, V.S., Vainchtein, I.D., Molofsky, A.V., and Kheirbek, M.A. (2020). Circuit and molecular architecture of a ventral hippocampal network. Nat. Neurosci. *23*, 1444–1452. https://doi.org/10.1038/s41593-020-0705-8.
79. Felix-Ortiz, A.C., Beyeler, A., Seo, C., Leppla, C.A., Wildes, C.P., and Tye, K.M. (2013). BLA to vHPC inputs modulate anxiety-related behaviors. Neuron *79*, 658–664. https://doi.org/10.1016/j.neuron.2013.06.016.
80. Jimenez, J.C., Su, K., Goldberg, A.R., Luna, V.M., Biane, J.S., Ordek, G., Zhou, P., Ong, S.K., Wright, M.A., Zweifel, L., et al. (2018). Anxiety Cells in a Hippocampal-Hypothalamic Circuit. Neuron *97*, 670–683.e6. https://doi.org/10.1016/j.neuron.2018.01.016.
81. Jimenez, J.C., Berry, J.E., Lim, S.C., Ong, S.K., Kheirbek, M.A., and Hen, R. (2020). Contextual fear memory retrieval by correlated ensembles of ventral CA1 neurons. Nat. Commun. *11*, 3492. https://doi.org/10.1038/s41467-020-17270-w.
82. Beyeler, A., Namburi, P., Glober, G.F., Simonnet, C., Calhoon, G.G., Conyers, G.F., Luck, R., Wildes, C.P., and Tye, K.M. (2016). Divergent Routing of Positive and Negative Information from the Amygdala during Memory Retrieval. Neuron *90*, 348–361. https://doi.org/10.1016/j.neuron.2016.03.004.
83. Canteras, N.S., Simerly, R.B., and Swanson, L.W. (1992). Connections of the posterior nucleus of the amygdala. J. Comp. Neurol. *324*, 143–179. https://doi.org/10.1002/cne.903240203.
84. Canteras, N.S., and Swanson, L.W. (1992). Projections of the ventral subiculum to the amygdala, septum, and hypothalamus: a PHAL anterograde tract-tracing study in the rat. J. Comp. Neurol. *324*, 180–194. https://doi.org/10.1002/cne.903240204.
85. Jackson, A.D., Cohen, J.L., Phensy, A.J., Chang, E.F., Dawes, H.E., and Sohal, V.S. (2024). Amygdala-hippocampus somatostatin interneuron beta-synchrony underlies a cross-species biomarker of emotional state. Neuron *112*, 1182–1195.e5. https://doi.org/10.1016/j.neuron.2023.12.017.
86. Sprengelmeyer, R., Young, A.W., Schroeder, U., Grossenbacher, P.G., Federlein, J., Büttner, T., and Przuntek, H. (1999). Knowing no fear. Proc. Biol. Sci. *266*, 2451–2456. https://doi.org/10.1098/rspb.1999.0945.
87. Fanselow, M.S., and Kim, J.J. (1994). Acquisition of contextual Pavlovian fear conditioning is blocked by application of an NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid to the basolateral amygdala. Behav. Neurosci. *108*, 210–212.
88. Rumpel, S., LeDoux, J., Zador, A., and Malinow, R. (2005). Postsynaptic receptor trafficking underlying a form of associative learning. Science *308*, 83–88. https://doi.org/10.1126/science.1103944.
89. Han, J.H., Kushner, S.A., Yiu, A.P., Hsiang, H.L.L., Buch, T., Waisman, A., Bontempi, B., Neve, R.L., Frankland, P.W., and Josselyn, S.A. (2009). Selective erasure of a fear memory. Science *323*, 1492–1496. https://doi.org/10.1126/science.1164139.
90. Ribeiro, A.M., Barbosa, F.F., Munguba, H., Costa, M.S.M.O., Cavalcante, J.S., and Silva, R.H. (2011). Basolateral amygdala inactivation impairs learned (but not innate) fear response in rats. Neurobiol. Learn. Mem. *95*, 433–440. https://doi.org/10.1016/j.nlm.2011.02.004.
91. Tye, K.M., Prakash, R., Kim, S.Y., Fenno, L.E., Grosenick, L., Zarabi, H., Thompson, K.R., Gradinaru, V., Ramakrishnan, C., and Deisseroth, K. (2011). Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature *471*, 358–362. https://doi.org/10.1038/nature09820.
92. Moreira, C.M., Masson, S., Carvalho, M.C., and Brandão, M.L. (2007). Exploratory behaviour of rats in the elevated plus-maze is differentially sensitive to inactivation of the basolateral and central amygdaloid nuclei. Brain Res. Bull. *71*, 466–474. https://doi.org/10.1016/j.brainresbull.2006.10.004.
93. Bueno, C.H., Zangrossi, H., and Viana, M.B. (2005). The inactivation of the basolateral nucleus of the rat amygdala has an anxiolytic effect in the elevated T-maze and light/dark transition tests. Braz. J. Med. Biol. Res. *38*, 1697–1701. https://doi.org/10.1590/s0100-879x2005001100019.
94. Bang, J.Y., Sunstrum, J.K., Garand, D., Parfitt, G.M., Woodin, M., Inoue, W., and Kim, J. (2022). Hippocampal-hypothalamic circuit controls context-dependent innate defensive responses. Elife *11*, e74736. https://doi.org/10.7554/eLife.74736.
95. Yan, J.J., Ding, X.J., He, T., Chen, A.X., Zhang, W., Yu, Z.X., Cheng, X.Y., Wei, C.Y., Hu, Q.D., Liu, X.Y., et al. (2022). A circuit from the ventral subiculum to anterior hypothalamic nucleus GABAergic neurons essential for anxiety-like behavioral avoidance. Nat. Commun. *13*, 7464. https://doi.org/10.1038/s41467-022-35211-7.
96. Ozawa, T., Ycu, E.A., Kumar, A., Yeh, L.F., Ahmed, T., Koivumaa, J., and Johansen, J.P. (2017). A feedback neural circuit for calibrating aversive

memory strength. Nat. Neurosci. *20*, 90–97. https://doi.org/10.1038/nn.4439.

97. Rescorla, R.W., and Wagner, A.R. (1972). A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. In Classical Conditioning: II. Current Research and Theory, A.H. Black and W.F. Prokasy, eds., pp. 64–99.
98. Anagnostaras, S.G., Wood, S.C., Shuman, T., Cai, D.J., Leduc, A.D., Zurn, K.R., Zurn, J.B., Sage, J.R., and Herrera, G.M. (2010). Automated assessment of pavlovian conditioned freezing and shock reactivity in mice using the video freeze system. Front. Behav. Neurosci. *4*, 158. https://doi.org/10.3389/fnbeh.2010.00158.
99. Pennington, Z.T., Diego, K.S., Francisco, T.R., LaBanca, A.R., Lamsifer, S.I., Liobimova, O., Shuman, T., and Cai, D.J. (2021). ezTrack-A Step-by-Step Guide to Behavior Tracking. Curr. Protoc. *1*, e255. https://doi.org/10.1002/cpz1.255.
100. Pennington, Z.T., Dong, Z., Feng, Y., Vetere, L.M., Page-Harley, L., Shuman, T., and Cai, D.J. (2019). ezTrack: An open-source video analysis pipeline for the investigation of animal behavior. Sci. Rep. *9*, 19979. https://doi.org/10.1038/s41598-019-56408-9.
101. Lattal, K.M., and Abel, T. (2001). Different requirements for protein synthesis in acquisition and extinction of spatial preferences and context-evoked fear. J. Neurosci. *21*, 5773–5780. https://doi.org/10.1523/JNEUROSCI.21-15-05773.2001.
102. Frankland, P.W., Ding, H.K., Takahashi, E., Suzuki, A., Kida, S., and Silva, A.J. (2006). Stability of recent and remote contextual fear memory. Learn. Mem. *13*, 451–457. https://doi.org/10.1101/lm.183406.
103. Lattal, K.M., and Abel, T. (2004). Behavioral impairments caused by injections of the protein synthesis inhibitor anisomycin after contextual retrieval reverse with time. Proc. Natl. Acad. Sci. USA *101*, 4667–4672. https://doi.org/10.1073/pnas.0306546101.
104. Grecksch, G., and Matthies, H. (1980). Two sensitive periods for the amnesic effect of anisomycin. Pharmacol. Biochem. Behav. *12*, 663–665. https://doi.org/10.1016/0091-3057(80)90145-8.
105. Wanisch, K., and Wotjak, C.T. (2008). Time course and efficiency of protein synthesis inhibition following intracerebral and systemic anisomycin treatment. Neurobiol. Learn. Mem. *90*, 485–494. https://doi.org/10.1016/j.nlm.2008.02.007.
106. Flood, J.F., Rosenzweig, M.R., Bennett, E.L., and Orme, A.E. (1973). The influence of duration of protein synthesis inhibition on memory. Physiol. Behav. *10*, 555–562. https://doi.org/10.1016/0031-9384(73)90221-7.
107. Franklin, K., and Paxinos, G. (2008). The Mouse Brain in Stereotaxic Coordinates, 3 Edition (Elsevier Inc.).
108. Ko, B., Yoo, J.Y., Yoo, T., Choi, W., Dogan, R., Sung, K., Um, D., Lee, S.B., Kim, H.J., Lee, S., et al. (2023). Npas4-mediated dopaminergic regulation of safety memory consolidation. Cell Rep. *42*, 112678. https://doi.org/10.1016/j.celrep.2023.112678.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Polyclonal rabbit anti cFos	Synaptic Systems	226 003
Goat anti rabbit Alexa Fluor 568	Invitrogen	A-11036
Bacterial and virus strains		
AAV5-hSyn-hM4Di-mCherry	Armbruster et al., 20071	Addgene: 50475
AAV5-hSyn-eGFP	Bryan Roth, MD, PhD	Addgene: 50465
AAV5-hSyn-DIO-HM4Di-mCherry	Armbruster et al., 20071	Addgene: 44362
AAV5-hSyn-DIO-mCherry	Bryan Roth, MD, PhD	Addgene: 50459
AAVrg-ef1a-Cre	Fenno et al., 20142	Addgene: 55636
AAV8-hSyn-eGFP	Bryan Roth, MD, PhD	Addgene: 50465
AAV5-hSyn-PSAM4-GlyR-IRES-EGFP	Magnus et al., 2019[76]	Addgene: 119742
Chemicals, peptides, and recombinant proteins		
Anisomycin from *Streptomyces griseolus*	Millipore Sigma	Millipore Sigma: A9789
CNO-dihydrochloride	Tocris	Tocris: 6329
uPSEM 817 tartrate	Tocris	Tocris: 6866
Experimental models: Organisms/strains		
Mouse: C57BL/6J	The Jackson Laboratory	JAX: 000664
Software and algorithms		
Med Associates Video Freeze	Med Associates[98]	Med Associates: SOF-843
ezTrack	Pennington et al., 2019[100]	www.github.com/denisecailab/eztrack
Automated cell counting	Zachary Pennington, PhD	www.github.com/zachpenn/cellcounting

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

All animals were adult C57BL/6J mice obtained from Jackson Laboratories, aged 2–6 months. A mix of male and female mice were used to demonstrate the initial effects of stress on subsequent defensive behavior (Figure 1), as well as the systemic effects of protein synthesis inhibition (Figure 2). Because no sex differences were observed, all other experiments were performed using males. Two animals were excluded from behavioral analyses after they spent >95% time in the light in the light-dark test (Figure 1). All other exclusions were due to inaccurate viral/cannula placement. Animals were housed in a temperature- and humidity-controlled vivarium on a 12/12 light-dark cycle (lights on at 7 a.m.), and all handling and behavioral testing took place during the light phase. All experimental procedures were approved by the Icahn School of Medicine at Mount Sinai's IACUC.

METHOD DETAILS

Behavioral testing

For all experiments, animals were singly housed beginning 1 week prior to the start of behavioral testing and were handled by the experimenters for approximately 1 min/day for 5 days during this time. When systemic injections were to be given, animals were additionally briefly habituated to restraint 2–3 times. Animals were habituated to being transported from the vivarium to the laboratory 2–3 times to mitigate transport serving as an associative cue.

Trauma and trauma recall

Animals were transported from the vivarium in their cages on a cart to the experimental testing room, which was well lit and had an air filter providing ambient sound. Animals were then placed in a brightly lit experimental testing chamber with a grid floor (Med Associates), scented with 5% Simple Green solution. During trauma, after a 5 min period of baseline exploration, animals received 10, 1 s, 1 mA, scrambled footshocks, with an inter-shock interval of 30 s. Animals were taken out of the testing chamber 30 s after the last shock and returned to the vivarium in their home cage. For trauma recall sessions, animals were transported to the same experimental testing chamber for an 8 min test session.

Novel stressor and novel stressor recall

Animals were transported from the vivarium in P1000 pipet boxes and carried in a dark cardboard box to the experimental testing room, which was dark except for a dim red light. Animals were then placed inside of a dark testing chamber (Med Associates) with a flat plexiglass floor and a curved back wall. The chamber was scented with 1% acetic acid solution. After a 3 min baseline period, animals were exposed to a single loud auditory stimulus (3 s, 130 dB white noise, 0 ms rise time) that was delivered by a speaker attached to the wall. Animals were removed 10 s later and returned to the vivarium. For novel stressor recall sessions, animals were transported to the same experimental testing chamber for an 8 min test session.

Exploratory anxiety-related tests

The light-dark test was conducted using two interconnected square compartments with an open top (each compartment measured 7.5 in width x 11.25 in height), separated by a 1.5 in wide passageway that could be closed with an opaque sliding divider. One chamber was made of all white acrylic, while the walls of the other were covered in matte black wallpaper and had a red acrylic floor. Overhead lighting provided luminance of 50 lux on the light side. After a 1 min baseline period in which animals were confined to the dark side, the central divider was raised and the animals could freely explore both sides of the light-dark box. The open field test was conducted in a circular arena (19 in diameter; 10 in height) made of white acrylic. A circular field was utilized in order to avoid the need to define arbitrary center/outer areas. Animals were placed along one wall and allowed to explore for 5 min. Luminance was approximately 50 lux. For the elevated plus maze, each arm measured 2 3/8 in wide and 13.75 in long. The floor of the maze was made of white acrylic, and the enclosed arms had black walls (8 in high). Luminance of the open arms was 25 lux. Animals were placed at the end of a closed arm and then allowed to explore freely for 5 min. For all anxiety-related behavior tests, apparatus were cleaned with 70% ethanol between test sessions and behavior was captured with an overhead webcam. These sessions were conducted in an otherwise dark room with a fan providing ambient background noise.

Learning rate analysis

To examine differences in the acquisition of associative fear after trauma (Figure S2), animals experienced the same 10 footshock trauma described above, or were placed in the same environment but received no shocks. The next day, mice were placed back in the trauma context for an 8 min context test. Beginning the following day, all animals were placed in a novel environment and received one weak shock per day across 7 days (Footshock = 2 s, 0.25 mA. 3 min baseline. Taken out 30 s after shock. Note that this is the same environment across these 7 days). A low amplitude footshock was utilized because it is known to produce lower asymptotic freezing levels.[96] Data is compared qualitatively to predictions from the Rescorla-Wager Model.[97] In addition to looking at the percentage of time spent freezing, we also examined how animals progressed toward asymptotic freezing levels. For each animal, asymptote was defined by their average level of freezing across days 3–7. We then calculated freezing on each day as a percent of asymptotic freezing. Lastly, we assessed shock reactivity evoked by the low intensity shock in the novel environment. For each animal, average baseline motion prior to shock onset, as well as average motion during the shock, was ascertained across the seven days of conditioning. Group differences in shock-induced motion were then assessed.

Behavior quantification

For analysis of freezing and motion in conditioning chambers, Med Associates Video Freeze software was used to analyze videos acquired from a near infra-red camera located in the chamber.[98] For measuring distance traveled and time spent in regions of interest in anxiety-related behavior tests, ezTrack was used.[99,100] With the exception of freezing during the trauma and novel stressor session, all measures reflect the average across the entire session. For freezing during the trauma, time was binned into the 300 s baseline and then 10 post-shock periods. Each post-shock period was 20 s in length and began 10 s after shock offset.

Surgery

For surgery, anesthesia was induced with 5% isoflurane and subsequently maintained at 1–2%. Body temperature was maintained during surgery and recovery with a heating pad below the animal, and ophthalmic ointment was applied to lubricate the eyes. All surgeries followed aseptic surgical technique. For viral surgeries, 100–150 nL was infused into the BLA (AP: −1.4; ML: 3.3; DV: −5) or vHC (AP: −3; ML: 3.2; DV: −4.5) at 2 nL/s via glass pipettes. For pan-neuronal HM4D experiments, either 100 nL of AAV5-hSyn-HM4Di-mCherry (7 x 10^{12} GC/mL; Addgene 50475) or AAV5-hSyn-eGFP (2.26 x 10^{12}; Addgene 50465) was infused. For projection-specific HM4D experiments, 150 nL containing a cocktail of AAVrg-ef1a-Cre (final concentration = 1.1 x 10^{13} GC/mL; Addgene 55636) and AAV8-hSyn-eGFP (final concentration = 3.8 x 10^{12} GC/mL; Addgene 50465) were infused into the projection target structure. Additionally, 150 nL of AAV5-hSyn-DIO-HM4Di-mCherry (2.4 x 10^{13} GC/mL; Addgene 44362), or AAV5-hSyn-DIO-mCherry (7.3 x 10^{12} GC/mL; Addgene 50459), was infused into the projection origin structure. For PSAM experiments, 100 nL of AAV5-hSyn-PSAM4-GlyR-IRES-EGFP (2.4 x 10^{13} GC/mL; Addgene 119742) was infused. Alternatively, an equivalent volume of sterile PBS was infused. 10 min was allowed for diffusion before removing the injector, irrigating the incision with saline, and suturing the incision site. For cannulation surgeries, 26 gauge guide cannula (P1 Technologies; 8IC315GMNSPC) were implanted overlying the BLA (AP: −1.4; ML: 3.2; DV: −3.5) or vHC (AP: −3; ML: 3.2; DV: −3), and affixed to the skull with dental cement and super glue. A skull screw was also implanted during surgery to help secure the head cap (P1 Technologies; 00-96X1/16). After surgery, dummy cannula that extended 1.5 mm below the guide cannula were inserted (P1 Technologies 8IC315DCMNSP). Following surgery, animals were given 20 mg/kg ampicillin and 5 mg/kg carprofen (s.c.) per day for 7 days and body weight and general disposition were monitored.

Anisomycin experiments

For experiments in which anisomycin (Sigma A9789) was administered systemically, we utilized a dose of 150 mg/kg (10 mL/kg, s.c.), consistent with prior literature.[101,102] Because numerous waves of protein synthesis have been found to support memory consolidation,[68,103,104] we opted to administer anisomycin 3 times, once every 4 h. In line with prior reports,[105,106] this should maintain approximately 90% blockade of protein synthesis for 12 h. Control animals treated with vehicle were injected/infused at the same times as animals receiving anisomycin (saline for systemic injections; PBS for intracranial injections). For experiments in which anisomycin was administered intracranially, 33 gauge injectors (P1 Technologies; 8IC315IMNSPC) attached via PE-20 tubing (Instech) to a Harvard syringe pump (Harvard Apparatus, #55–2222) were utilized to infuse anisomycin (10 ng/nL) at a rate of 150 nL/min 300 nL of anisomycin solution was administered per hemisphere in the vHC. 200 nL was administered per hemisphere in the BLA. Control animals were infused with an equivalent volume of 1X PBS. Critically, we used a dose of anisomycin that was unable to affect memory recall (Figure S4), but was nevertheless able to alter translation of the immediate-early gene cFos (Figure S4). Moreover, although the dosing regimen used was found to alter memory consolidation when given immediately after a learning event, it had no long-term deleterious impacts on future learning (Figure S4). Following infusions, injectors were left in place for 1 min before removal. Again, anisomycin was infused 3 times, once every 4 h. Prior to testing, animals were habituated to handling such that infusions could be done while mice were gently held by the experimenter. Additionally, all animals received a habituation infusion of 1X PBS 2–3 days prior to the trauma day. Anisomycin was first dissolved in a small volume of 0.1 N HCL (90% PBS, 10% 1 N HCL), brought near concentration with the addition of 1X PBS, and the pH was then normalized to 6–7 by the addition of 1 N NaOH.

HM4D experiments

For HM4D experiments, actuation of HM4D was achieved through intraperitoneal administration of 3 mg/kg cno-dihydrochloride (Tocris), 30–40 min prior to behavior, at a volume of 10 mL/kg (dissolved in saline). For the study in which the effects of inhibiting the vHC or BLA were assessed on multiple measures of exploratory anxiety-related behavior, each animal underwent each of these tests twice, once with cno and once with vehicle, in a counterbalanced order. Tests occurred in a counterbalanced order, with the constraint that each test (EPM, open field, light-dark) was experienced once before that test was repeated.

PSAM experiments

For PSAM experiments, actuation of PSAM4-GlyR was achieved through intraperitoneal administration of 1 mg/kg uPSEM-817-tartrate (Tocris), 15–20 min prior to behavior, at a volume of 10 mL/kg (dissolved in saline). For the study in which the effects of inhibiting the vHC or BLA were assessed on multiple measures of exploratory anxiety-related behavior, each animal underwent each of these tests twice, once with uPSEM and once with vehicle. Tests occurred in a fixed order across two weeks, with open-field on Monday, EPM on Wednesday, and Light-Dark on Friday. However, drug order was counterbalanced, such that half the animals that received uPSEM on the first open field test received saline on the first EPM, and so forth.

Histology

At the end of behavioral testing, animals that underwent surgical manipulation were deeply anesthetized, and their brains were then extracted and placed in paraformaldehyde overnight at 4C. For animals with cannula implants, 100 nL of DAPI (0.5 mg/mL) was infused prior to brain extraction, but after anesthesia, to mark cannula placement. The next day, brains were transferred to 30% sucrose in 1X PBS and left at 4C to sink before being frozen and sectioned at 50 μm on a cryostat. Tissue was then mounted on slides and either cover-slipped using mounting media with DAPI (Vector Laboratories, #H-1200-10) for checking viral placement or cover-slipped with non-fluorescent mounting media (Vector Laboratories, #H-1000-10) after a green nucleic acid stain. For green nucleic acid staining, slides were submerged in 50 mM Sytox Green (diluted in 1X PBS from 5 mM. Thermo Fisher #S7020) for 10 min and then washed 3x in 1X PBS. Tissue was then imaged on a Leica DM6 epifluorescent microscope. Viral expression and cannula placement was evaluated using the mouse brain atlas of Franklin and Paxinos.[107]

Ex vivo electrophysiology

Acute coronal brain slices were prepared in slice cutting solution (20 mM NaCl, 3.5 mM KCl, 1.4mM NaH2PO4, 26 mM NaHCO3, 11 mM D-glucose, 175 mM sucrose, 1.3 mM MgCl2) at a thickness of 350 μm. All recordings for BLA and vHC principal neurons were performed with a MultiClamp 700B amplifier (Molecular Devices) in artificial cerebrospinal fluid solution (119 mM NaCl, 2.5 mM CaCl2, 2.5 mM KCl, 1.25 mM NaH2PO4, 2 mM MgSO4, 26 mM NaHCO3, and 10 mM D-glucose, equilibrated with 95% O2 and 5% CO2 at pH 7.2–7.4).[108] Fluorescence signal was identified under a Nikon Eclipse FN1 microscope (Nikon Instrument Inc.) using SOLA light engine (Lumencor). Cell-attached recordings were performed in voltage clamp mode. The baseline firing rate was measured 10 min prior to bath application of clozapine N-oxide (CNO, 20 μM). The firing rate was then continuously measured over 50 min of CNO application. Average firing rate during the last 10 min were then compared to the baseline firing rate. Data was analyzed using Clampfit 10.7 software (Molecular Devices).

Immunohistochemistry and image analysis

Immunohistochemistry for cFos was performed on 50 μm free-floating tissue sections. In brief, tissue was blocked for 1 h in a mixture of 0.3% Triton X-100 and 3% normal goat serum in 1X PBS. Tissue was then incubated in a solution of primary antibody plus blocking

solution overnight (Synaptic Systems 226 003, polyclonal rabbit anti cFos, 1:2000), followed by incubation in secondary antibody for ~4 h (Invitrogen A-11036, goat-anti rabbit Alexa Fluor 568, 1:500). Tissue was washed 3 times, 10 min each, before and after incubation in secondary. All steps were performed at room temperature with gentle shaking. Lastly, tissue was mounted on slides and coverslipped using mounting media with DAPI.

Images were acquired at 10x on a Leica DM6B microscope, using identical exposure settings across animals. For each animal, 4–5 image tiles of the entire amygdala were collected.

c-Fos counts were performed in an automated manner utilizing custom in-house code (https://github.com/ZachPenn/CellCounting). In brief, a median filter was applied to each image to remove granular noise, local fluctuations in background fluorescence were removed with a large Gaussian kernel, and a binary threshold was then applied to separate cells from background. A watershed algorithm was implemented to separate adjacent cells and individual cell puncta were then counted. All parameters were applied in an equivalent manner to all images and selected based upon concordance with a set of manually counted images. Regions of interest were drawn and the number of cFos positive cells per unit area was determined.

QUANTIFICATION AND STATISTICAL ANALYSIS

All analyses were performed using RStudio. All data and statistical analysis are available at github.com/ZachPenn/2024_CellReports. Group sizes are listed in each figure legend. Briefly, omnibus ANOVA were conducted using the package ezANOVA with type 3 degrees of freedom. The white adjustment was implemented to correct for heterogeneity of variance using heteroscedasticity corrected standard errors ('hc3'). For repeated measures ANOVA, the Greenhouse-Geisser correction was implemented when the assumption of sphericity was not met. Post-hoc t-tests and planned comparisons did not assume equal variance between groups (Welch test). Post-hoc t-tests were only conducted following main effects/interactions at omnibus significance, but omnibus tests are not reported in the main text for the sake of clarity. Detailed statistics with omnibus results can be found in Table S1. Post-hoc tests and planned comparisons were evaluated against a modified criterion calculated using the Dunn-Sidak method in order to keep family-wise type 1 error at 0.05. Criterion p values for significance can be found in Table S1. F and t values are rounded to the nearest tenth and hundredth, respectively. Where F values were less than .1, F is listed as 0.