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# Endocannabinoid Signaling Collapse Mediates Stress-Induced Amygdalo-Cortical Strengthening

# **Highlights**

- The BLA-pIPFC circuit is engaged by stress exposure and its activation is anxiogenic
- Stress enhances glutamate release in a reciprocal BLApIPFC-BLA subcircuit
- BLA-pIPFC glutamatergic drive is constrained by multimodal 2-AG signaling
- 2-AG signaling collapse mediates stress-induced circuit strengthening and anxiety

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# In Brief

Marcus et al. show that acute stress exposure induces a persistent collapse of multimodal endocannabinoid-mediated inhibition of a reciprocal BLA-pIPFC-BLA glutamatergic subcircuit and that this collapse is causally related to the generation of stress-induced anxiety-like behaviors.





# Endocannabinoid Signaling Collapse Mediates Stress-Induced Amygdalo-Cortical Strengthening

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### SUMMARY

Functional coupling between the amygdala and the dorsomedial prefrontal cortex (dmPFC) has been implicated in the generation of negative affective states; however, the mechanisms by which stress increases amygdala-dmPFC synaptic strength and generates anxiety-like behaviors are not well understood. Here, we show that the mouse basolateral amygdala (BLA)-prelimbic prefrontal cortex (pIPFC) circuit is engaged by stress and activation of this pathway in anxiogenic. Furthermore, we demonstrate that acute stress exposure leads to a lasting increase in synaptic strength within a reciprocal BLA-pIPFC-BLA subcircuit. Importantly, we identify 2-arachidonoylglycerol (2-AG)-mediated endocannabinoid signaling as a key mechanism limiting glutamate release at BLA-pIPFC synapses and the functional collapse of multimodal 2-AG signaling as a molecular mechanism leading to persistent circuitspecific synaptic strengthening and anxiety-like behaviors after stress exposure. These data suggest that circuit-specific impairment in 2-AG signaling could facilitate functional coupling between the BLA and pIPFC and the translation of environmental stress to affective pathology.

# INTRODUCTION

Stress exposure is a major risk factor for the development and exacerbation of mental illnesses ranging from major depression to anxiety and substance use disorders (Arnsten, 2015; McEwen, 2012; Sharma et al., 2016). Furthermore, exposure to severe stress is required for the development of posttraumatic stress disorder (PTSD) (Fenster et al., 2018; Gillespie et al.,

2009; Henigsberg et al., 2019; Kessler et al., 2005; Mark et al., 2018). In this context, understanding the molecular, cellular, and circuit-level mechanisms by which stress exposure is translated into distinct pathological behavioral, emotional, and cognitive domains could have broad translational implications. Moreover, elucidating conserved molecular mechanisms linking stress to affective psychopathology could reveal novel therapeutic approaches to mitigate the adverse effects of stress on mental health. Although the identification of a number of stress-regulatory neuromodulatory signaling systems has revealed potentially novel therapeutic targets for affective disorders, endogenous cannabinoid (eCB) signaling represents a leading drug-development candidate (McCormick et al., 2017; Hill et al., 2018; Hill and Patel, 2013; Lowe et al., 2019; Patel et al., 2017).

eCB signaling systems have been heavily implicated in stressresponse physiology, and pharmacological augmentation of eCB signaling has been suggested to represent a novel approach for the treatment of stress and trauma-related psychiatric disorders (Hill et al., 2018; Patel et al., 2017). At the synaptic level, 2-arachidonoylglycerol (2-AG)-mediated eCB signaling is a broadly expressed inhibitory retrograde signaling system. Specifically, 2-AG is canonically produced by postsynaptic neurons in an activity-dependent manner by diacylglycerol-lipase alpha (DAGLa) and activates presynaptic CB1 receptors to reduce neurotransmitter release probability (Kano et al., 2009; Stella et al., 1997; Wilson and Nicoll, 2001). 2-AG is degraded by monoacylglycerol lipase (MAGL) expressed within presynaptic terminals and glial cells (Dinh et al., 2004). Recent studies have implicated 2-AG signaling as a critical stress modulatory system and suggested 2-AG augmentation as a novel approach for the treatment of stress-related psychiatric disorders (Lisboa et al., 2017; Lutz et al., 2015). For example, 2-AG deficiency is associated with increased anxiety, impaired fear extinction, and increased susceptibility to stress-induced anxiety (Bluett et al., 2017; Cavener et al., 2018; Shonesy et al., 2014). Conversely, 2-AG augmentation promotes stress resilience and prevents stress-induced anxiety (Bedse et al., 2017; Bluett et al., 2017;

Bosch-Bouju et al., 2016; McLaughlin et al., 2014; Patel et al., 2009; Qin et al., 2015; Sciolino et al., 2011; Sumislawski et al., 2011). Despite these data, the precise cellular and circuit-level mechanisms by which 2-AG interacts with environmental stress to affect emotional behavior are not well understood.

Studies over the past decade have elucidated distinct brain circuits connecting emotional and cognitive control centers in the modulation of stress responsivity, anxiety, and emotional regulation (Apps and Strata, 2015; McEwen et al., 2015; Tovote et al., 2015; Tye, 2018). For example, in humans, the dorsomedial prefrontal cortex (dmPFC) and amygdala show excitatory coupling during exposure to threatening stimuli, and enhanced activity in the amygdala-dmPFC circuit is correlated with subjective ratings of anxiety (Carlisi and Robinson, 2018; Cremers et al., 2010; Kim et al., 2011; Milad et al., 2009; Robinson et al., 2014). Consistent with these findings, activation of rodent basolateral amygdala (BLA) inputs to the prelimbic PFC (pIPFC), a rodent homolog of the human dmPFC, generates anxiety-like behaviors and biases behavior toward fear responses in the face of uncertainty (Burgos-Robles et al., 2017; Felix-Ortiz et al., 2016; Senn et al., 2014). Recent studies have also demonstrated that BLApIPFC glutamatergic synapses undergo presynaptic strengthening after stress exposure (Lowery-Gionta et al., 2018). Taken together, these data suggest that enhanced amygdala-dmPFC (BLA-pIPFC in rodents) coupling could represent a conserved circuit mechanism that translates stress exposure into anxietylike emotional states. However, the molecular mechanisms subserving stress-induced strengthening of BLA-pIPFC circuits and generation of anxiety-like behaviors after stress exposure are not known. Here, we elucidate an eCB mechanism linking stress exposure to BLA-pIPFC subcircuit-specific synaptic strengthening and persistent anxiety-like behavior.

# RESULTS

# The BLA-pIPFC Circuit Is Stress Responsive and Anxiogenic

To investigate the molecular mechanisms regulating BLA-pIPFC connectivity and plasticity, we first verified that this circuit is engaged by exposure to stress (Figure 1A). Using in vivo fiber photometry, we observed that unpredictable foot-shock stress significantly increased presynaptic Ca2+ influx in BLA axon terminals innervating pIPFC, time-locked to shock onset (Figures 1B and 1C). We next used in vivo single-cell Ca<sup>2+</sup> imaging to examine pIPFC neuronal responses to foot-shock exposure. The bulk Ca<sup>2+</sup> signal in the entire field of view was increased in response to shock exposure (Figures 1D and 1E), with subsequent single-cell analysis revealing three distinct populations of neurons: stress excitatory (44.40%), stress inhibitory (38.43%), and stress non-responsive (17.16%) (Figures 1F and 1G). The peak excitatory response was greater in absolute magnitude than the peak inhibitory response (excitatory: |z| =3.83, inhibitory: |z| = 2.62, p = 0.0073 by two-tailed t test), and the resultant average signal was excitatory (Z = 1.09; Figure 1H), suggesting that stress-induced excitation of pIPFC neurons predominates over inhibition. These data indicate that stress exposure engages the BLA-pIPFC circuit and leads into enhanced activity of pIPFC neurons.

Stress exposure is a ubiquitous risk factor for the development of anxiety disorders, and stress exposure in rodents can model many psychopathological domains relevant to affective disorders. Indeed, 24 h following foot-shock stress exposure, we observed an increase in anxiety-like behavior in the elevatedzero maze (EZM) (Figure 1I). To test whether a "stress-like" state could be recapitulated through direct activation of the BLApIPFC circuit, we used an intersectional chemogenetic approach to specifically enhance the excitability of BLA neurons that project to the pIPFC (Figure 1J). We used whole-cell currentclamp electrophysiology to show that pIPFC-projecting BLA pyramidal neurons that express the hM3D Designer Receptor Exclusively Activated by Designer Drugs (GqDREADD) fire action potentials (APs) following clozapine N-oxide (CNO) application (Figure 1K). Administration of CNO also induced robust cFOS expression in both the BLA (Figure 1L) and the mPFC (Figure 1M). Furthermore, although stress led to neuronal activation in the pIPFC (Figures 1D and 1E), stress did not lead to a further increase in cFOS expression in the BLA (-n = 6, p = 0.6418) or pIPFC (n = 6, p = 0.0855) after GqDREADD activation of BLApIPFC neurons, suggesting that stress and GqDREADD activation recruit overlapping BLA-pIPFC neural circuits (data not shown). In the elevated-plus maze (EPM), chemogenetic activation of this circuit significantly enhanced anxiety-like behavior (Figure 1N). These data indicate that the BLA-pIPFC circuit is stress responsive and its activation is anxiogenic in mice, suggesting that stress-induced anxiety-like behavior could be mediated by enhancement of BLA-pIPFC circuit function.

# Stress Exposure Potentiates Excitatory Signaling in a BLA-pIPFC Reciprocal Circuit

Our data thus far suggest that enhanced BLA-pIPFC circuit activity may be a relevant substrate for the translation of environmental stress into anxiety-like behaviors. To examine stressinduced synaptic adaptations within the BLA-pIPFC circuit, we used a combination of anterograde ChR2-assisted projection-targeting, retrograde tracing approaches, and ex vivo electrophysiology (Figures 2A and 2B). Four to six weeks after co-injection of an anterograde adeno-associated virus AAV5-CaMKII-ChR2-eYFP (ChR2) and a retrograde AAV2-CAG-td-Tomato (rAAV) into the BLA, mice were sacrificed for ex vivo electrophysiological studies. Monosynaptic BLA-originating, optically evoked excitatory post-synaptic currents (oEPSCs) were recorded from rAAV-positive (rAAV+) and rAAV-negative (rAAV-) pyramidal neurons in both L2/3 and L5 in the pIPFC (Figures 2 and S1A-S1C). Consistent with previous studies, we found BLA inputs made stronger synaptic connections onto reciprocally projecting rAAV+ pIPFC neurons than non-reciprocally projecting rAAV- neurons (Figures S1D-S1I) (Little and Carter, 2013).

24 h after foot-shock exposure, we observed an increase in BLA-originating oEPSC amplitude and a decrease in the paired-pulse ratio (PPR) specifically in rAAV+ L2/3 pIPFC neurons (Figures 2C–2E), suggesting increased glutamate release probability within a reciprocal BLA-pIPFC-BLA subcircuit after stress. This enhanced excitatory drive was accompanied by an increase in the probability of synaptically driven action potential firing in rAAV+ L2/3 neurons and a small but significant increase in the intrinsic excitability of these neurons (Figures 2F and 2G).



# Figure 1. Stress Exposure Activates an Anxiogenic BLA-pIPFC Circuit

(A) Schematic and timeline for stress exposure and behavioral analysis.

(B) Schematic for *in vivo* fiber photometry recordings of BLA projections to the pIPFC.

(C) Z score of  $\Delta$ F/F signal recorded from GCaMP6s expressing BLA terminals in the pIPFC in response to a 2-s 0.5-mA footshock. Traces represent mean of 4–5 mice, with each mouse trace derived from the average of 20 shock trials (stress: n = 5, control: n = 4).

(D) Schematic for *in vivo* miniendoscopy-based Ca<sup>2+</sup> imaging of pIPFC neurons.

(E) Z score of bulk ΔF/F recorded from total GCaMP7f signal in the field of view in response to a 2-s 0.5-mA foot shock. Trace represents mean of 4 mice, with each mouse trace derived from the average of 20 shock trials (n = 4).

(F) Miniendoscopic maximal projection image of Ca<sup>2+</sup> signal and extracted cell contours from representative mouse.

(G) Proportion of GCaMP7f-expressing pIPFC neurons displaying excitatory, inhibitory, or no responses to foot shock (n = 268 cells from 4 mice).

(H) Z score of excitatory (n = 119), inhibitory (n = 103), and averaged excitatory and inhibitory responses (n = 222), across 1-s bins. Peak excitatory Z score = 3.83, peak inhibitory Z score = -2.14, peak average = 1.09.

(I) Effects of foot-shock stress on anxiety-like behavior in the elevated-zero maze (no stress: n = 10, stress: n = 15). % open-arm time (p < 0.0001), open-arm entries (p < 0.0001), total distance (p < 0.0001), and immobility time (p = 0.0003).

(J) Schematic of intersectional viral approach for hM3Dq (GqDREADD) expression in the BLA-pIPFC circuit.

(K) Example trace and proportion of BLA neurons that displayed CNO-induced action potential firing (n = 13, 4 mice).

(L) Representative images and quantification of cFOS expression in BLA neurons following *in vivo* administration of 10 mg/kg CNO (mCherry: n = 8, GqDREADD: n = 8). mCherry versus GqDREADD, p < 0.0001.

(M) Representative images and quantification of cFOS expression in pIPFC neurons following *in vivo* administration of 10 mg/kg CNO (mCherry: n = 8, GqDREADD: n = 8). mCherry versus GqDREADD, p < 0.0001.

(N) Effects of CNO (5 mg/kg) on anxiety-like behavior in the elevated-plus maze (EPM) (GqDREADD: n = 9, mCherry: n = 8). % open+center time (p = 0.0378), open+center entries (p = 0.0268), total distance (p = 0.0067), and immobility time (p = 0.0119).

All error bars represent ± SEM; n represents number of neurons or number of mice in behavioral studies. p values reported from two-tailed unpaired t test (I, L, M, and N).



#### Figure 2. Stress Exposure Enhances Glutamatergic Signaling in the BLA-L2/3 pIPFC Reciprocal Circuit (A) Experimental timeline

(B) Schematic for stereotaxic delivery of AAV5-CaMKII-ChR2(H147R)-eYFP and rAAV2-CAG-td-Tomato into the BLA and recordings of oEPSCs from

retrogradely labeled pIPFC neurons. (C) Schematic for voltage-clamp recordings of oEPSCs from L2/3 rAAV-positive (rAAV+) neurons.

(D) Optically evoked input/output curve from L2/3 rAAV+ neurons from nonstressed (n = 11, N = 4) and stressed (n = 11, N = 4 mice) mice.

(E) Effects of stress on paired pulse ratio (PPR) at BLA-L2/3 rAAV+ synapses (no stress: n = 11, 4 mice; stress: n = 10, 4 mice).

(F) Intrinsic excitability of L2/3 rAAV+ neurons following stress exposure (no stress: n = 37, 12 mice; stress: n = 33, 14 mice).

(G) Optically evoked spiking in L2/3 rAAV+ neurons in non-stressed (n = 20, 7 mice) and stressed mice (n = 19, 7 mice).

(H) Effects of stress on asynchronous EPSC frequency (no stress: n = 12, 4 mice; stress: n = 12, 5 mice; p = 0.0394) and amplitude (not significant [NS], p = 0.4311) at BLA-L2/3 rAAV+ synapses.

(I) Schematic for voltage-clamp recordings of oEPSCs from L2/3 rAAV-negative (rAAV-) neurons.

(J) Optically evoked input/output curve from L2/3 rAAV- neurons from nonstressed (n = 9, 4 mice) and stressed (n = 9, 4 mice) mice.

(K) Effects of stress on PPR at BLA-L2/3 rAAV- synapses (no stress: n = 8, 4 mice; stress: n = 9, 4 mice).

(L) Intrinsic excitability of L2/3 rAAV– neurons following stress exposure (no stress n = 35, 11 mice; stress n = 28, 10 mice).

(M) Optically evoked spiking in L2/3 rAAV – neurons in non-stressed (n = 17, 7 mice) and stressed mice (n = 19, 7 mice).

All error bars represent  $\pm$  SEM; n represents number of neurons. p values reported from two-tailed unpaired t test (H). F and p values for two-way ANOVA are shown in relevant panels.

We also observed an increase in the frequency, but not amplitude, of optogenetically elicited asynchronous EPSCs (o-aEPSCs) onto L2/3 rAAV+ neurons, confirming an increase in presynaptic release probability at BLA-pIPFC synapses 24 h after stress exposure (Figure 2H). In contrast, there was no significant change in excitatory input (Figure 2J), PPR (Figure 2K), or somatically driven AP firing (Figure 2L) in L2/3 rAAV- neurons after stress. The only change observed in L2/3 rAAV- neurons was a significant decrease in optogenetically elicited action potential firing (Figure 2M).

No stress-induced changes were found in L5 rAAV+ or rAAVneurons (Figures S2A–S2H), suggesting that both subcircuit-(BLA-pIPFC reciprocal versus non-reciprocal circuits) and laminar-specific synaptic strengthening occurs after acute stress exposure. Additionally, no stress-induced changes in either resting membrane potential or input resistance were found in any population of neurons (Figures S2I–S2T). Lastly, to determine the specificity of stress-induced synaptic strengthening in the pIPFC, we examined whether projections from the mediodorsal thalamus (MDT) were modulated by stress exposure (Mátyás et al., 2014; Vertes, 2004). We observed that stress did not cause persistent alterations at MDT to L2/3 or to L6 synapses, suggesting that there is selective enhancement of excitatory signaling in the BLA-L2/3 pIPFC-BLA reciprocal circuit (Figures S2U–S2d).

Similar effects were observed in male mice exposed to the predator odor analog 2-methyl-thiazoline (2MT) and in footshock-exposed female mice, suggesting that stress-induced enhancement of presynaptic glutamate release at BLA-pIPFC-BLA synapses is not modality or sex specific (Figures S3A–S3C,

S3E, and S3F). We next determined whether stress-induced presynaptic strengthening at BLA-L2/3 pIPFC-BLA synapses was dependent on stressor intensity. Fiber photometry approaches revealed an increase in BLA-pIPFC terminal activity at a 0.25, but not a 0.1, mA shock intensity (Figures S3H–S3J). Similarly, 24 h later, we observed a trend toward increased oEPSC amplitude and significantly reduced PPR in the 0.25-mA, but not in the 0.1-mA, shock group, suggesting that stress-induced BLA-pIPFC terminal activation and persistent strengthening both scale dynamically with the intensity of the stressor (Figures S3K and S3L). Lastly, to determine the duration of these synaptic modifications, we also performed electrophysiological recordings at 3 and 10 days following exposure to 20 0.5-mA foot shocks. At 3 days poststress, oEPSC amplitudes remained higher and PPR remained reduced, whereas these values were normalized 10-days poststress, indicating that these stress-induced synaptic alterations are relatively persistent, but not permanent (Figures S3N and S3O).

# Endocannabinoid Signaling Broadly Inhibits Glutamatergic Input from the BLA to the pIPFC

Given that the observed stress-induced increase in BLA-pIPFC glutamatergic transmission is mediated by enhanced presynaptic release, we next sought to determine the mechanism driving this effect. The retrograde acting eCBs, namely anandamide (AEA) and 2-AG, are known to be regulated by stress and modulate synaptic transmission in the PFC, raising the possibility that functional impairment in this neuromodulatory system could contribute to increased presynaptic drive at BLA-pIPFC synapses after stress exposure (Katona and Freund, 2012; Lafourcade et al., 2007; Manduca et al., 2017; McLaughlin et al., 2014). To test this hypothesis, we first demonstrated that the cannabinoid receptor agonist CP55,940 robustly depressed BLA-evoked oEPSC amplitude in rAAV+ and rAAV- L2/3 pIPFC neurons, indicating that BLA projections to the pIPFC are broadly regulated by cannabinoid receptors (Figures 3A, 3B, 3E, and 3F). To determine whether eCBs regulate BLA-pIPFC glutamatergic transmission, we analyzed depolarization-induced suppression of excitation (DSE), a well-characterized form of 2-AG-mediated short-term synaptic depression, in which brief post-synaptic depolarization leads to 2-AG production and inhibition of glutamate release through binding to presynaptic CB1 receptors (Kano et al., 2009; Wilson and Nicoll, 2001). We found that DSE, induced by 10-s post-synaptic depolarization to +30mV, was expressed at both L2/3 rAAV+ and rAAV- neurons, and could be blocked by rimonabant, a CB1 inverse agonist, or DO34, an inhibitor of the rate-limiting enzyme in 2-AG biosynthesis, diacylglycerol lipase (DAGL) (Figures 3C and 3G). Interestingly, these two compounds alone induced a dramatic reduction in the PPR, suggesting tonic inhibition of glutamate release at BLApIPFC synapses by 2-AG-CB1 signaling (Figures 3D and 3H). Similar results were found in L5 rAAV+ and rAAV- neurons, although DSE could not be elicited efficiently in L5 rAAV- neurons (Figures S4A–S4H). To ensure that the effect of rimonabant on PPR was due to blockade of tonic eCB signaling and not its inverse agonist properties, we repeated this experiment with the neutral CB1 antagonist NESS0327 (NESS). Both NESS and rimonabant blocked DSE, reduced the PPR, and led to greater oEPSC amplitude, further supporting tonic eCB control over BLA-pIPFC glutamatergic synapses (Figures S5J-S5M, S5P, and S5Q). Given that tonic eCB signaling has generally been ascribed to AEA, rather than 2-AG (Kim and Alger, 2010), we augmented our PPR experiments by demonstrating that both rimonabant and DO34 increased the frequency, but not amplitude, of o-aEPSCs, confirming tonic 2-AG-CB1 signaling at these synapses (Figures 3I-3K). These data provide evidence for broadly expressed multimodal tonic and phasic 2-AG-mediated regulation of presynaptic neurotransmitter release at BLApIPFC synapses. To examine the input specificity of eCB signaling in the pIPFC, we performed the same key experiments while examining MDT projections to the pIPFC. The MDT is known to have low CB1 expression (Herkenham et al., 1990), and as such, we observed little CP55,940-induced depression of oEPSC amplitude and no DSE, suggesting there is circuit-level specificity to eCB modulation of excitatory transmission in the pIPFC (Figures S4I-S4K).

# Stress Impairs 2-AG Inhibition of the BLA-pIPFC Reciprocal Glutamatergic Circuits

Following the observation that stress increased presynaptic release probability within the BLA-pIPFC reciprocal circuit and that BLA inputs to the pIPFC are highly regulated by 2-AG signaling, we next sought to determine whether stress-induced synaptic strengthening within this circuit was mediated by dynamic remodeling of BLA-pIPFC 2-AG signaling. Using mass spectrometry, we observed that stress exposure decreased 2-AG levels in the mPFC 24 h later, consistent with previous studies in other brain regions (Qin et al., 2015) (Figure 4A). Furthermore, bulk levels of arachidonic acid (AA), a primary degradative product of 2-AG hydrolysis, were also significantly reduced in the mPFC of stressed mice (Figure 4B). These data suggest that stress exposure could downregulate 2-AG synthesis in the mPFC, as levels of both 2-AG and AA are similarly reduced following stress exposure: in support of this, inhibition of 2-AG hydrolysis with the MAGL inhibitor JZL184 increased 2-AG and reduced AA (Figures 4A and 4B). Importantly, elevating 2-AG levels with JZL184 reversed, whereas depleting 2-AG levels with DO34 exacerbated stress-induced increases in anxiety-like behavior in the EZM, supporting the notion that 2-AG signaling can bidirectionally modulate stress-induced anxiety (Figure 4C). Lastly, we found the anxiolytic effects of JZL184 required CB1 receptor availability (Figure S5C).

We next tested the hypothesis that stress-induced strengthening of the BLA-pIPFC reciprocal circuit was mediated by the collapse of 2-AG-mediated inhibition of BLA-pIPFC glutamatergic transmission. We found once again that stress increased presynaptic release probability selectively onto L2/3 rAAV+ neurons, as indicated by a decrease in the PPR 24 h after stress exposure (Figures 4D and 4E). Interestingly, although bath application of DO34 reduced PPR in non-stressed mice, this effect was occluded in stressed animals. Furthermore, DO34 application occluded further stress-induced reductions in PPR. Conversely, pharmacological augmentation of 2-AG signaling by bath application of JZL184 was able to selectively rescue this stress-induced decrease in PPR at BLA-L2/3 rAAV+ synapses in a CB1-dependent manner (Figures 4E and S5A). Lastly,



### Figure 3. Phasic and Tonic 2-AG Signaling Broadly Regulate BLA-L2/3 pIPFC Glutamatergic Synapses

(A) Schematic for voltage-clamp recordings of oEPSCs from L2/3 rAAV+ neurons.

(B) Effect of 5 μM CP55,940 on oEPSC amplitude at BLA-L2/3 rAAV+ synapses (n = 6, 3 mice).

(C) Depolarization-induced suppression of excitation (DSE) at BLA-L2/3 rAAV+ synapses (n = 12, 6 mice) is blocked by 10  $\mu$ M rimonabant (n = 9, 5 mice; p = 0.0049) and 2.5  $\mu$ M DO34 (n = 8, 4 mice; p = 0.0039).

(D) PPR at BLA-L2/3 rAAV+ synapses (n = 13, 6 mice) is reduced by 10  $\mu$ M rimonabant (n = 10, 6 mice; p = 0.0034) and 2.5  $\mu$ M DO34 (n = 10, 4 mice; p = 0.0002). (E) Schematic for voltage-clamp recordings of oEPSCs from L2/3 rAAV- neurons.

(F) Effect of CP55,940 on oEPSC amplitude at BLA-L2/3 rAAV- synapses (n = 5, 4 mice).

(G) DSE at BLA-L2/3 rAAV - synapses (n = 10, 5 mice) is blocked by rimonabant (n = 10, 4 mice; p = 0.0095) and DO34 (n = 10, 4 mice; p = 0.0095).

(H) PPR at BLA-L2/3 negative synapses (n = 13, 5 mice) is reduced by rimonabant (n = 10, 5 mice; p = 0.0084) and DO34 (n = 9, 4 mice; p = 0.0409).

(I) Schematic for voltage-clamp recordings of asynchronous EPSCs from L2/3 rAAV+ neurons.

(J) Asynchronous EPSC frequency at BLA-L2/3 rAAV+ synapses (n = 9, 2 mice) is increased by rimonabant (n = 2 mice; p = 0.0058) and DO34 (n = 9, 2 mice; p = 0.0058).

(K) Asynchronous EPSC amplitude at BLA-L2/3 rAAV+ synapses (n = 9, 2 mice) is not altered by rimonabant (n = 10, 2 mice; p = 0.5392) or DO34 (n = 9, 2 mice; p = 0.5785). Representative traces for vehicle, rimonabant, and DO34 groups (right).

All error bars represent ± SEM; n represents number of neurons. All post hoc p values are derived from one-way ANOVA with Holm-Sidak multiple comparisons (C, D, G, H, J, and K). F and p values for ANOVA are shown in relevant panels.

a similar pattern was observed with regard to phasic 2-AG signaling, as stress reduced DSE magnitude in L2/3 rAAV+ neurons, an effect rescued by incubation with JZL184 (Figures 4F and 4G) and that was dependent upon stressor intensity and recovered 3 days after stress exposure (Figures S3M and S3P). As observed with the PPR, the effect of JZL184 on DSE could be blocked by rimonabant pre-application (Figure S5B). No stress-induced changes were observed in L2/3 rAAV- neurons (Figures 4H-4K) or L5 neurons (data not shown). These data suggest stress-induced collapse of phasic and tonic 2-AG signaling at BLA-pIPFC synapses could contribute to stress-induced BLA-pIPFC circuit strengthening and subsequent expression of anxiety-like behaviors (Figure 4L). Interestingly, female mice did not show a stress-induced reduction in DSE

magnitude, indicating that stress effects on tonic versus phasic 2-AG signaling could differ between sexes (Figure S3D). However, DSE was impaired by 2MT exposure, indicating that 2-AG signaling collapse is not stressor modality specific (Figure S3G). To further support stress-induced functional collapse of 2-AG-CB1 regulation of excitatory input from the BLA, we demonstrated that stress occludes the ability of rimonabant and NESS to increase oEPSC amplitude (Figures S5N and S5R) and fully occludes the ability of NESS to decrease the PPR while partially occluding the ability of rimonabant to decrease the PPR at BLA-L2/3 pIPFC rAAV+ synapses (Figures S5O and S5S).

Lastly, our mass spectrometry data indicated that AEA levels were also decreased in the mPFC following stress exposure



# Figure 4. Stress Impairs 2-AG Signaling within the BLA-L2/3 pIPFC Reciprocal Circuit

(A) Effect of stress and JZL184 on mPFC 2-AG levels. Stress exposure decreased mPFC 2-AG levels (no stress: n = 10; stress: n = 10; p = 0.0233). Treatment with 15 mg/kg JZL184 (n = 10) increased mPFC 2-AG levels in stressed mice (p < 0.0001).

(B) Effect of stress and JZL184 on mPFC AA levels. Stress exposure decreased mPFC levels of AA (no stress: n = 10; stress: n = 10, p = 0.0290). Treatment with 15 mg/kg JZL-184 (n = 10) reduced mPFC AA levels in stressed mice (p < 0.0001).

(C) Effect of stress on anxiety-like behavior in the EZM. Stress exposure decreased % time spent in the open arms (p = 0.0110) and open-arm entries (p = 0.0099; no stress: n = 10; stress: n = 10). 15 mg/kg JZL184 (n = 10) reversed the stress-induced decrease in % open-arm time (p = 0.0012) and open-arm entries (p = 0.0278). 50 mg/kg DO34 exacerbated the stress-induced decrease in % open-arm time (p = 0.0077) and open-arm entries (p < 0.0001).

(D) Schematic for voltage-clamp recordings of oEPSCs from L2/3 rAAV+ neurons.

(E) Effect of stress, 2.5  $\mu$ M DO34 or 1  $\mu$ M JZL on PPR at BLA-L2/3 rAAV+ synapses. Stress exposure decreased PPR (no stress: n = 21, 8 mice; stress: n = 27, 11 mice; p = 0.0053). DO34 decreased PPR in non-stressed mice (n = 10, 4 mice; p = 0.0008), but not in stressed mice (n = 10, 4 mice; p = 0.0904). JZL184 reversed the stress-induced decrease PPR (n = 13, 4 mice; p = 0.0075) but did not affect PPR in non-stressed mice (n = 12, 4 mice; p = 0.4778). (F) Effect of stress on DSE at BLA-L2/3 rAAV+ synapses.

(G) Effect of stress, DO34, and JZL184 on maximum DSE at BLA-L2/3 rAAV+ synapses. Stress exposure impaired DSE (no stress: n = 18, 7 mice; stress: n = 24, 11 mice; p = 0.0072). DO34 blocked DSE in both non-stressed (n = 10, 4 mice; p < 0.0001) and stressed (n = 9, 4 mice; p = 0.0129) mice. JZL184 selectively reversed the stress-induced impairment of DSE (n = 11, 4 mice; p = 0.0312). (H) Schematic for voltage-clamp recordings of oEPSCs from L2/3

rAAV- neurons. (I) Effect of stress, DO34, and JZL184 on PPR at BLA-L2/3-rAAVsynapses. Stress exposure did not alter PPR (no stress: n = 15, 8mice; stress: n = 11, 7 mice; p = 0.9249). DO34 significantly decreased PPR in both non-stressed (n = 9, 4 mice; p = 0.0451) and stressed (n = 8, 4 mice; p = 0.0035) mice.

(J) Effect of stress on DSE at BLA-L2/3 rAAV- synapses.

(K) Effect of stress, DO34, and JZL184 on maximum DSE at BLA-L2/3 rAAV- synapses. Stress exposure did not alter DSE (no stress: n = 16, 8 mice; stress: n = 14, 7 mice; p = 0.9476). DO34 blocked DSE in both non-stressed (n = 9, 4 mice; p = 0.0155) and stressed (n = 9, 4 mice; p = 0.0035) mice.

(L) Stress impairs 2-AG regulation of BLA-L2/3 pIPFC-BLA synapses, leading to strengthening of BLA-L2/3 pIPFC reciprocal circuits involved in generating anxiety-like responses by activation of BLA output neurons to the ventral hippocampus (vHIPP) or central amygdala (CeA), for example.

All error bars represent ± SEM; n represents number of neurons or mice in biochemical and behavioral studies. All post hoc p values are derived from one-way ANOVA (A, B, and C) or two-way ANOVA (E, G, I, and K) with Holm-Sidak multiple comparisons. F and p values for ANOVA are shown in relevant panels.



Figure 5. pIPFC-Specific DAGL a Deletion Phenocopies Synaptic and Behavioral Effects of Stress

(A) Immunohistochemical validation of pIPFC conditional knockout of DAGLa; AAV-Cre-GFP or AAV-GFP injection (green) and DAGLa expression (red).

(B) Schematic approach for electrophysiological examination of BLA-pIPFC circuit after pIPFC-specific DAGLa deletion.

(C) Effect of pIPFC DAGLa deletion on PPR at BLA-L2/3 pIPFC rAAV+ synapses (wild-type [WT]: n = 11, 5 mice; DAGLa<sup>frf</sup>: n = 7, 3 mice; p = 0.0002).

(D) Effect of pIPFC DAGLα deletion on DSE at BLA-L2/3 pIPFC rAAV+ synapses (WT: n = 14, 6 mice; DAGLα<sup>frf</sup>: n = 7, 3 mice; p = 0.0026).

(E) Effect of pIPFC DAGL $\alpha$  deletion on anxiety-like behavior in the EZM. Mice with pIPFC DAGL $\alpha$  deletion (n = 9) show decreased % open-arm time (p = 0.0213), decreased open-arm entries (p = 0.0197), and similar distance traveled (p = 0.2006) compared to GFP-injected controls (n = 10).

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(Figure S5D). This hints at the possibility that stress could also impair AEA regulation of the BLA-pIPFC circuit. However, incubation with an inhibitor of AEA degradation, PF3845, affected neither basal PPR nor rescued the stress-induced decrease in PPR or DSE magnitude, suggesting that AEA does not strongly regulate BLA-pIPFC glutamatergic transmission (Figures S5E–S5G). Furthermore, stress exposure did not affect cannabinoid agonist-induced synaptic depression at BLA-L2/3 pIPFC synapses, suggesting stress impairs 2-AG levels rather than affecting CB1 receptor sensitivity (Figures S5H and S5I).

# Prelimbic DAGLa Deletion Increases BLA-pIPFC Synaptic Strength and Anxiety-like Behavior

Our data thus far indicate that 2-AG plays a crucial role in limiting excitatory input from the BLA to the pIPFC and that stress exposure compromises the efficacy of this signaling in a circuitspecific manner, contributing to synaptic strengthening after stress. These data suggest 2-AG signaling deficiency within the pIPFC could contribute to the generation of anxiety-like behaviors by enabling enhanced BLA-pIPFC glutamatergic coupling after stress. To address this hypothesis experimentally, we took advantage of conditional DAGLa knockout mice (Bluett et al., 2017). We first demonstrated that stereotaxic injection of AAV-Cre into the pIPFC of DAGL $\alpha^{f/f}$  mice resulted in selective reduction in the DAGLa protein in the pIPFC but not the adjacent infralimbic PFC (iIPFC) (Figure 5A). Using pIPFC-specific DAGLa knockout combined with the aforementioned injections of ChR2 and rAAV2-td-Tomato into the BLA, we found significantly impaired DSE and a robust decrease in the PPR at BLA-pIPFC glutamatergic synapses, recapitulating the synaptic phenotype observed after stress exposure (Figures 5B-5D). Consistent with this stress-like synaptic phenotype, pIPFC-specific DAGLa deletion elicited an anxiety-like behavioral phenotype that was similar to the anxiogenic effects observed after stress exposure (Figure 5E). This behavioral profile persisted following exposure to stress, as examined in a separate cohort of mice, suggesting a crucial role for pIPFC 2-AG signaling in regulating anxiety-like behavior (Figure 5F). These data suggest that impaired pIPFC 2-AG signaling results in a stress-like synaptic phenotype at BLA-pIPFC glutamatergic synapses and is sufficient to induce anxiety-like behavior, recapitulating the effects of stress exposure (Figure 5G). Taken together, our data support the hypothesis that stress-induced impairment in 2-AG signaling at BLA-pIPFC synapses could represent an important mechanism translating the effects of stress into anxiety-like behavior.

# Circuit-Specific CB1 Deletion Increases Synaptic Strength and Stress-Induced Anxiety-like Behavior

To further solidify the role of 2-AG-CB1 signaling specifically at BLA-pIPFC synapses in the regulation of stress-induced anxiety,

we utilized an intronic recombinase sites enabling combinatorial targeting (INTRSECT) approach to selectively delete the CB1 receptor from BLA neurons projecting to the pIPFC (Fenno et al., 2017). Using a mouse in which exon 2 of *Cnr1* is flanked by loxP sites (CB1<sup>f/f</sup>; Figures S6A–S6E), we injected a retrograde virus that drives the expression of FIp recombinase and td-Tomato into the pIPFC and a virus that drives the expression of Cre recombinase in a Flp-dependent manner into the BLA. Injection of both of these viruses into CB1<sup>f/f</sup> mice would be predicted to delete the CB1 receptor specifically from pIPFC-projecting BLA neurons (Figures 6A and 6E). Using electrophysiological approaches, we first showed that this approach leads to almost complete functional removal of CB1 from BLA neurons projecting to the pIPFC, as oEPSCs are completely insensitive to the cannabinoid agonist CP55,940 compared to wild-type mice injected with the same viral combinations (Figure 6B). The circuit selectivity of this manipulation was verified by demonstrating intact cannabinoid receptor function at BLA-nucleus accumbens synapses after the BLA-pIPFC-specific CB1 deletion (Figures S6F-S6H).

Similar to pIPFC DAGLa deletion, we found that BLA-pIPFCspecific CB1 deletion resulted in a synaptic phenotype comparable to that observed after stress exposure, as evidenced by a lower PPR and reduced DSE magnitude (Figures 6C and 6D). We next determined the behavioral consequences of BLApIPFC-specific CB1 deletion at baseline and after stress exposure (Figures 6E and 6F). Prior to stress exposure, BLApIPFC-specific CB1 deletion did not affect anxiety-like behavior in the EZM compared to control virus (pIPFC rAAV-td-Tomato combined with fDIO-Cre-mNeonGreen in the BLA)-injected CB1<sup>f/f</sup> littermates (Figure 6G). However, deletion of CB1 from the BLA-pIPFC pathway enhanced anxiety-like behavior 24 h after foot-shock stress exposure in the EPM in the same cohort of mice, suggesting a pivotal role for this CB1 population in regulating stress-induced anxiety-like behavior (Figure 6H). Together, these data support our global hypothesis that stressinduced strengthening of BLA-pIPFC circuits is mediated by circuit-specific impairment in 2-AG-CB1 signaling (Figure 6I).

# DISCUSSION

The amygdala and dmPFC are crucial components of the negative valence system in humans, and their functional connectivity is integral to threat reactivity (Carlisi and Robinson, 2018; Kalisch and Gerlicher, 2014; Robinson et al., 2014). Here, we demonstrate circuit-specific 2-AG signaling collapse at BLA-pIPFC synapses links environmental stress exposure to anxiety-like behavior in mice. Specifically, we found that stress caused a selective strengthening of glutamatergic transmission within a reciprocal BLA-pIPFC-BLA subcircuit, which was driven by

<sup>(</sup>F) Effect of pIPFC DAGL $\alpha$  deletion on anxiety-like behavior in the EZM 24 h following stress exposure. Mice with pIPFC DAGL $\alpha$  deletion (n = 9) show decreased % open-arm time (p = 0.0339), decreased open-arm entries (p = 0.0322), and similar distance traveled (p = 0.0941) compared to GFP-injected controls (n = 10) following stress exposure.

<sup>(</sup>G) Deletion of DAGLα from pIPFC neurons reduces 2-AG supression of BLA input to pIPFC neurons, induces a stress-like synaptic phenotype, and results in increased anxiety-like behavior.

All error bars represent ± SEM; n represents number of neurons or number of mice in behavioral studies. p values reported from two-tailed unpaired t test (C, D, E, and F).



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the collapse of multimodal retrograde 2-AG-mediated eCB signaling. Importantly, experimentally induced circuit-specific impairment in BLA-pIPFC 2-AG-CB1 signaling phenocopied both stress-induced circuit strengthening and persistent anxiety-like behavior. These studies support the critical role of the BLA-pIPFC circuit in the generation of anxiety-like states after stress and reveal an eCB-mediated mechanism subserving stress-induced BLA-pIPFC circuit strengthening and behavioral adaptation. Our data are consistent with the notion that the amygdala and dmPFC comprise an "aversive amplification circuit" associated with elevated threat processing during stress (Robinson et al., 2014) and the anxiogenic effects of BLA-pIPFC circuit activation in mice (Felix-Ortiz et al., 2016; Lowery-Gionta et al., 2018). Taken together, these data suggest that aberrant activity and/or plasticity in this circuit could contribute to the pathogenesis of stress-related and affective disorders and that increased amygdala-dmPFC connectivity/ signaling represents a conserved translationally relevant mechanism linking environmental stress to its behavioral, emotional, and cognitive consequences.

It is important to note that the BLA-pIPFC circuit is not a closed loop. Indeed, both the BLA and pIPFC send excitatory projections to brain regions that are directly involved in invigorating behavioral responses. For example, BLA-projecting pIPFC neurons, in addition to synapsing onto reciprocally projecting BLApIPFC neurons, also strongly synapse onto open-loop BLA projection neurons innervating the ventral hippocampus and central amygdala (McGarry and Carter, 2017), providing a potential mechanism whereby the BLA-pIPFC-BLA reciprocal circuit gains access to limbic output structures to generate behavioral responses to stress. Supporting this hypothesis, optogenetic activation of BLA-ventral hippocampal projections increases anxiety and induces social avoidance in mice (Felix-Ortiz et al., 2013).

Although there is a prominent role of prefrontocortical eCB signaling in regulating presynaptic glutamate release and anxiety-like behaviors (Lafourcade et al., 2007; Lisboa et al., 2014; Lutz et al., 2015; Manduca et al., 2017; Puente et al., 2011; Rubino et al., 2008), whether this modulation is ubiquitous or circuit specific is not known. Our data indicate that CB1 receptors and retrograde 2-AG signaling are present at BLA-pIPFC but not

MDT-pIPFC synapses, suggesting that 2-AG regulates pIPFC glutamatergic transmission in a circuit-specific manner. Interestingly, our data indicate that 2-AG modulates BLA-pIPFC glutamatergic transmission in both phasic and tonic modes. Specifically, although prototypical phasic 2-AG signaling in the form of DSE is present at BLA-pIPFC synapses and blocked by inhibitors of CB1 receptors and DAGL, both inhibitors also decrease the PPR and increase the frequency of BLA-elicited asynchronous EPSCs, revealing tonic inhibition of BLA-pIPFC glutamatergic transmission by 2-AG-CB1 signaling. Take together with our data that BLA-pIPFC-specific CB1 deletion and a conditional pIPFC DAGL $\alpha$  deletion also decrease PPR, these data strongly indicate that 2-AG exerts both tonic and phasic presynaptic inhibition of BLA-pIPFC glutamatergic transmission.

Given that that BLA projections to the pIPFC are multimodally inhibited by 2-AG signaling and that some forms of stress impair eCB signaling (Bluett et al., 2017; Hill et al., 2009; McLaughlin et al., 2012; Patel et al., 2004; Rademacher et al., 2008; Wamsteeker et al., 2010), we next determined whether 2-AG signaling collapse could drive the stress-induced strengthening of BLApIPFC glutamatergic synapses. In support of this hypothesis, we found impairment in both tonic and phasic 2-AG modulation of BLA to L2/3 pIPFC reciprocally projecting neuron synapses. This impairment was occluded by DAGL and CB1 inhibition and could be reversed by pharmacological inhibition of MAGL, which increases 2-AG levels, but not by inhibition of FAAH, which selectively increases AEA levels. Paralleling these data, systemic MAGL inhibition reduced, while DAGL inhibition increased, stress-induced anxiety-like behaviors. Given these findings, in tandem with our data demonstrating that activation of the BLA-pIPFC circuit is sufficient to induce anxiety-like behavior, we suggest that 2-AG-CB1 signaling collapse within the BLApIPFC-BLA reciprocal circuit could represent a mechanistic link between stress exposure, BLA-pIPFC synaptic strengthening, and stress-induced anxiety-like behaviors.

Previous studies have shown that prolonged stress exposure can compromise eCB signaling by reductions in CB1 receptor signaling and/or expression (Hill et al., 2005; Hillard, 2014; Patel et al., 2009; Wamsteeker et al., 2010). However, our acute stress manipulations did not affect CB1 receptor function at BLA-pIPFC synapses. Therefore, the most likely mechanism

(D) Effect of BLA-pIPFC CB1 deletion on DSE at BLA-L2/3 pIPFC synapses (WT: n = 6, 2 mice; CB1<sup>t/f</sup>: n = 6, 3 mice; p = 0.0010).

(E) Schematic of INTRSECT approach for deletion of the CB1 receptor from pIPFC-projecting BLA neurons for behavioral characterization using CB1<sup>f/f</sup> mice. (F) Representative images showing injection site of rAAV2-EF1-FIp-td-Tomato in the pIPFC and AAV5-fDIO-Cre-mNeonGreen in the BLA and co-expression of rAAV+ neurons (red) and Cre+ neurons (green) in the BLA (boxed area).

(G) Effect of BLA-pIPFC CB1 deletion on anxiety-like behavior in the EZM in stress-naive mice. There were no significant differences in anxiety-like behavior in Flpinjected CB1<sup>t/f</sup> mice (n = 9) compared to td-Tomato-injected CB1<sup>t/f</sup> mice (n = 10).

(I) Deletion of CB1 from BLA projections to the pIPFC induces a stress-like synaptic phenotype and exacerbates the anxiogenic effects of stress exposure. All error bars represent ± SEM; n represents number of neurons or number of mice in behavioral studies. p values reported from two-tailed unpaired t test (B, C, D, G, and H).

**Figure 6. BLA-pIPFC-Specific CB1 Deletion Phenocopies Stress-Induced Synaptic Strengthening and Exacerbates Stress-Induced Anxiety** (A) Schematic for physiological validation of the INTRSECT approach for deletion of the CB1 receptor from pIPFC-projecting BLA neurons using CB1<sup>*Vf*</sup> mice. (B) Effect of 5 μM CP55,940 on oEPSC amplitude at BLA-L2/3 pIPFC synapses. CP55,940-induced depression of oEPSC amplitude is dramatically attenuated in CB1<sup>*Vf*</sup> INTRSECT mice (n = 7, 3 mice) compared to WT control INRSECT mice (n = 6, 2 mice; p = 0.0028).

<sup>(</sup>C) Effect of BLA-pIPFC CB1 deletion on PPR at BLA-L2/3 pIPFC synapses (WT: n = 5, 2 mice;  $CB1^{t/t}$ : n = 7, 3 mice; p = 0.0026).

<sup>(</sup>H) Effect of BLA-pIPFC CB1 deletion on anxiety-like behavior in the EPM following stress exposure. Flp-injected CB1<sup>VI</sup> mice (n = 9) show significantly decreased % open-arm time (p = 0.0421) and decreased open-arm entries (p = 0.0391), without changes in total distance traveled (p = 0.1020), compared to control-virus-injected littermates (n = 10).

subserving 2-AG signaling collapse after acute stress is a reduction in 2-AG signaling availability, which could occur by reduced 2-AG synthesis or enhanced degradation. Our data support an impairment in 2-AG synthesis over changes in degradation, as both 2-AG and free AA levels were reduced and only the maximal magnitude (but not recovery) of DSE was reduced in the PFC 24 h after stress exposure. If increased 2-AG degradation was the primary driver of reduced 2-AG signaling, one would expect elevated levels of AA and a faster DSE decay (Pan et al., 2009; Zhong et al., 2014), neither of which were observed. However, additional mechanisms such as impaired 2-AG transport or combinatorial effects of multiple mechanisms cannot currently be excluded. Similarly, it is unclear from the present results what the initiating factors for 2-AG signaling collapse are. Although we hypothesize that the increase in the excitatory input from the BLA, and potentially other brain regions, during the stressor serves as the trigger that leads to a persistent reduction in 2-AG signaling capacity in reciprocally projecting L2/3 pIPFC neurons, this remains to be tested experimentally, and the subsequent molecular cascades resulting in impaired 2-AG signaling remain to be established. In addition, our single-cell calcium imaging and cFOS data suggest broad pIPFC neuronal activation after stress exposure, suggesting mechanisms must exist to guide the postsynaptic specificity of stress-induced 2-AG signaling impairment to BLA-pIPFC-BLA reciprocal circuit, which likewise remain to be elucidated.

To investigate whether BLA-pIPFC 2-AG signaling collapse is causally related to circuit strengthening and behavioral adaptations to stress, we used two different genetic strategies to impair BLA-pIPFC 2-AG-CB1 signaling. We hypothesized that because stress exposure reduces mPFC 2-AG levels and leads to anxietylike behavior, then direct depletion of pIPFC 2-AG, by pIPFCspecific DAGLa deletion, would recapitulate stress-induced BLA-pIPFC circuit strengthening and increase anxiety-like behavior. Consistent with this hypothesis, we found that pIPFC DAGLa deletion increased release probability at BLA-pIPFC synapses, impaired DSE, and elicited a corresponding increase in anxiety-like behavior, mimicking the effects of stress at the synaptic and behavioral level. Interestingly, a different behavioral pattern was observed following deletion of the CB1 receptor selectively from BLA neurons projecting to the pIPFC. Although this circuit-specific manipulation again phenocopied stressinduced synaptic strengthening, the deletion had a minimal effect on basal anxiety, but increased anxiety-like behavior following stress exposure. A possible explanation for the lack of robust behavioral effects of BLA-pIPFC CB1 deletion under basal conditions is that 2-AG-CB1 signaling plays a minimal role in regulating the activity of the quiescent circuit. However, following stressinduced activation of the BLA-pIPFC circuit, attenuation of 2-AG-CB1 negative feedback signaling impairs the ability of L2/3 pIPFC neurons to reduce excitatory input from the BLA, leading to an exacerbation of stress-induced anxiety-like behaviors. The differential effects of pIPFC DAGLa deletion and BLApIPFC-specific CB1 deletion in terms of effect on basal anxiety could also be explained by the fact that pIPFC DAGLa deletion impairs 2-AG signaling at all synapses (including other limbic inputs not examined here), resulting in a more robust behavioral phenotype. Future studies should be aimed at elucidating the roles of additional afferents and local eCB-sensitive circuits in the regulation of stress adaptation and anxiety-like behavior.

Here, we explored the neurobiological substrate by which stress exposure is translated into anxiety-like behavior and identified the collapse of 2-AG-CB1 signaling within a reciprocally connected BLA-pIPFC-BLA circuit as a molecular mechanism subserving stress-induced circuit strengthening and anxiety-like behavior. These data suggest that the enhancing 2-AG-CB1 signaling, by MAGL inhibition for example, could represent an attractive therapeutic approach for the treatment of stressinduced psychiatric disorders (Chanda et al., 2019; Lisboa et al., 2017; Patel et al., 2017). Furthermore, our data suggest that the analysis of functional connectivity in the amygdala-dmPFC circuit could represent a useful and conserved circuit-based biomarker bridging preclinical studies to MAGL inhibitor efficacy trials and could facilitate optimal patient selection for future clinical studies.

# **STAR**\***METHODS**

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#### SUPPLEMENTAL INFORMATION

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# **AUTHOR CONTRIBUTIONS**

D.J.M. conducted electrophysiological studies and contributed to experimental design, data analysis, and interpretation of results; D.J.M., A.D.G., and G.B. conducted behavioral and biochemical studies in the laboratory of S.P. J.D.R. conducted photometry experiments in the laboratory of F.S.L. E.D. generated CB1 floxed mice. V.K., N.D.W., and M.A. assisted with electrophysiological studies. L.E.R.-V. conducted the miniendoscopic recordings in the laboratory of S.P. K.M. supplied the DAGL $\alpha$  antibody. S.P. and D.J.M. are responsible for study conception, experimental design, and data interpretation. D.J.M. and S.P. wrote the manuscript with input from all authors.

#### **DECLARATION OF INTERESTS**

S.P. has received research support from H. Lundbeck A/S in the past 3 years. S.P. is a consultant for Psy Therapeutics.

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# **STAR**\***METHODS**

# **KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit-α-cFOS	Abcam	190289 RRID: AB_443538
Alexafluor 488 Donkey-α-Rabbit	Invitrogen	A21206 RRID: AB_221544
Rabbit-a-DAGLa	Gift from Ken Mackie	N/A
Bacterial and Virus Strains		
AAV5-CMV-fDIO-Cre-mNeonGreen-wPRE	This paper	VB180530-1030aad
rAAV2-CAG-tdTomato	Chan et al., 2017	RRID: Addgene_59462
AAV5-CaMKII-ChR2(H134R)-eYFP-wPRE	Lee et al., 2010	RRID: Addgene_26969
rAAV2-EF1a-mCherry-IRES-Flpo	Fenno et al., 2014	RRID: Addgene_55634
AAV5-CMV-Cre-eGFP-SV40	Gift from James M. Wilson	RRID: Addgene_105545
rAAV2-pmSyn1-EBFP-Cre	Madisen et al., 2015	RRID: Addgene_51507
AAV5-EF1a-DIO-ChR2(H134R)-eYFP-wPRE	Gift from Karl Deisseroth	RRID: Addgene_20298
AAV5-hSyn-DIO-hM3D(Gq)-mCherry	Krashes et al., 2011	RRID: Addgene_44361
rAAV2-hSyn-GCaMP7f-wPRE	Dana et al., 2016	RRID: Addgene_104488
Chemicals, Peptides, and Recombinant Proteins		
Rimonabant	Cayman Chemical	9000484
DO34	Glixx Laboratories	GLXC-09757
JZL184	Cayman Chemical	13158
CP55,940	Cayman Chemical	13608
PF3845	Cayman Chemical	13279
NESS0327	Cayman Chemical	10004184
CNO-HCI	Cayman Chemical	25780
Experimental Models: Organisms/Strains		
CB1 <sup>f/f</sup> mice	Dr. Eric Delpire	N/A
DAGL <sup>f/f</sup> mice	Dr. Sachin Patel	N/A
C57 WT mice	Jackson Laboratory	IMSR_JAX000664
Software and Algorithms		
Prism 6	Graphpad	https://www.graphpad.com
pClamp10.5	Molecular Devices	https://www.moleculardevices.com
MATLAB	Mathwords	https://www.mathworks.com
ANY-maze	Stoelting Co	https://stoelting.com/
Video Freeze	Med associates	https://www.med-associates.com
Other		
Fear Conditioning Chamber	Med associates	MED-VFC-SCT-M
Elevated Plus Maze	San Diego Instruments	N/A
Elevated Zero Maze	San Diego Instruments	N/A

# LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sachin Patel (sachin.patel@vanderbilt.edu). All unique/stable reagents generated in this study are available to academic researchers from the Lead Contact but may require completion of a Materials Transfer Agreement.

# **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

All experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committees and were conducted in accordance with the National Institute of Health guidelines for the Care and Use of Laboratory Animals. 8-14 week-old male and female C57BL/6J mice obtained from Jackson Labs were used for electrophysiological and behavioral experiments. 8-14 week-old male DAGL $\alpha^{t/f}$  and WT controls were used for electrophysiological and behavioral experiments in Figure 5. 10-18-week old male CB1<sup>f/f</sup> and WT controls were used for electrophysiological and behavioral experiments in Figure 6. Mice were housed in a temperature and humidity-controlled housing facility under a 12h light/dark cycle with *ad libitum* access to food. All behavioral and physiological experiments were run on littermate-matched stressed or non-stressed mice that had been singly housed  $\geq$  1 week. DAGL $\alpha^{t/f}$  and CB1<sup>f/f</sup> mice bred in house on a homozygous x homozygous breeding scheme, and littermate-matched controls were used for all behavioral experiments.

Littermate-matched mice were randomly assigned to treatment (e.g., stress versus no stress or virus versus control virus) for all behavioral and electrophysiological experiments performed, excluding the physiological validation for the DAGL $\alpha^{t/f}$  and CB1<sup>t/f</sup> knockouts. For behavioral cohorts, we used a homozygous/homozygous-breeding scheme, as preliminary reports from our lab and others that suggest that the CB1<sup>t/f</sup> mice have a basal phenotype in various behavioral assays that differentiates them from their wild-type littermates. Therefore, we used a homozygous/homozygous breeding scheme to generate only CB1<sup>t/f</sup> homozygotes, so that both the experimental and control groups had the same genetic background. However, this breeding scheme precluded our ability to perform physiological validation on littermate-matched controls, due to the fact that Cre expression would always lead to CB1 deletion in addition to allowing for expression of DIO ChR2. Therefore, we used non-littermate matched WT controls for physiological validation to determine whether the CB1 receptor or DAGL $\alpha$  was functionally deleted in the experimental group.

# Generation of CB1<sup>f/f</sup> mice

To produce a conditional knockout mouse of the cannabinoid receptor 1 (Cnr1 gene), we created a targeting construct centered around exon 2, the single coding exon of the gene (Figure S6). A first loxP site was inserted 141 bp upstream of the exon, whereas a DNA fragment containing a neomycin-resistance gene cassette flanked by frt and loxP sites was inserted 622 bp downstream of exon 2. Relatively large arms of recombination: 6.5 kb (5' end) and 3.1 kb (3' end) were then added to the construct (Figure S6). The cnr1 construct was electroporated into 129/SvEvTac embryonic stem cells and 540 neomycin resistant clones were picked and analyzed by Southern blot analysis. Genomic DNA was digested with Mfel, run on agarose gel, and transferred to nitrocellulose membranes to identify a 16 kb control fragment (wild-type allele) and a 7 kb mutant fragment (targeted allele) by Southern blot analysis. Out of the 540 clones, three clones were identified containing the mutant allele (Figure S6). Presence of the three loxP sites in ES cell clone 3H3 was verified by PCR and sequencing. The ES cell clone was injected into C57BL6 blastocysts which were implanted into pseudo-pregnant females to produce cnr1 chimeric mice. Three chimeras were produced from the 3H3 clone and one of these chimeras went germline and produced 9 pups, five of which carried the 3 loxP allele. After 2 backcrossing into C57BL6/J mice, the neomycin-resistance gene cassette was successfully removed by breeding a 3 loxP male mouse with two females carrying a FIpE allele (Figure S6). Functionality of the remaining 2 loxP sites was demonstrated by crossing a male carrying an E2a-CRE allele with two females carrying a cnr1 2 loxP allele. Seven out of 16 pups carried the E2a-CRE allele and out of them, four pups demonstrated successful elimination of exon 2 (Figure S6). The Cnr1 line was then backcrossed to C57BL/6J mice for an additional 5-6 generations prior to breeding to homozygosity.

# DAGL<sup>f/f</sup> mice

See Bluett et al. (2017).

# **METHOD DETAILS**

# **Surgeries**

Mice were initially anesthetized with 5% isoflurane and then transferred to the stereotax (Kopf Instruments, Tujunga, CA) and kept under 2% isoflurane anesthesia. The hair over the incision cite was trimmed and the skin was prepped with alcohol and iodine scrub. The skull was exposed via a midline sagittal incision and treated with the local anesthetic, benzocaine (Medline Industries, Brentwood, TN). For all surgeries, we used a motorized digital software (NeuroStar; Stoelting Co., Wood Dale, IL) to guide a 10  $\mu$ L microinjection syringe (Hamilton Co., Reno, NV) driven by a Micropump Controller (World Precision Instruments, Sarasota, FL). Virus was delivered bilaterally into the pIPFC (AP:+2.42, ML: ± 0.35, DV: 2.09), BLA (AP: -1.25, ML: ± 3.30, DV: 5.10), or MDT: (AP:-1.1, ML.: ± 0.59, DV: 3.4). Following completion of each surgery, 10mg/kg ketoprofen (AlliVet, St. Hialeah, FL) was administered as an analgesic, and post-operative treatment with ketoprofen was administered 24 and 48 hours after the surgery.

# Ex vivo Electrophysiology

Coronal brain slices were prepared at 250 μM on a vibrating Leica VT1000S microtome using standard procedures. Mice were anesthetized using isoflurane, and transcardially perfused with ice-cold and oxygenated cutting solution consisting of (in mM): 93 N-Methyl-D-glucamine (NMDG), 2.5 KCL, 20 HEPES, 10 MgSO4·7H20, 1.2 NaH2PO4, 0.5 CaCl2·2H20, 25 glucose,  $3 \text{ Na}^+$ -pyruvate,  $5 \text{ Na}^+$ -ascorbate, and 5 N-acetylcysteine. Following collection of coronal sections, the brain slices were transferred to a  $34^{\circ}\text{C}$  chamber containing oxygenated cutting solution for a 10-minute recovery period. Slices were then transferred to a holding chamber consisting of (in mM) 92 NaCl, 2.5 KCl, 20 HEPES,  $2 \text{ MgSO4} \cdot 7\text{H20}$ , 1.2 NaH2PO4, 30NaHCO3,  $2 \text{ CaCl} 2 \cdot 2\text{H20}$ , 25 glucose, 3 Na-pyruvate, 5 Na-ascorbate, 5 N-acetylcysteine and were allowed to recover for  $\geq 30 \text{ min}$ . For recording, slices were placed in a perfusion chamber (Warner Instruments RC-27L) and perfused with oxygenated artificial cerebrospinal fluid (ACSF;  $31-33^{\circ}\text{C}$ ) consisting of (in mM): 113 NaCl, 2.5 KCl, 1.2 MgSO4  $\cdot 7\text{H20}$ , 2.5 CaCl2  $\cdot 6\text{H20}$ , 1 NaH2PO4, 26 NaHCO3, 20 glucose,  $3 \text{ Na}^+$ -pyruvate, 1 Na $^+$ -ascorbate, at a flow rate of 2-3ml/min. All drugs were stored in DMSO stocks and then included in ACSF containing 1:2000 (w/v) Bovine Serum Albumin (Fisher Scientific) and  $\leq 1:2000$  (v/v) DMSO.

Fluorescently labeled neurons in the mPFC were identified using a series 120Q X-cite lamp at 40X magnification with an immersion objective with differential interference contrast microscopy (DIC). The pIPFC was visually distinguished from the iIPFC by packing density of L2/3 neurons and termination of BLA projections to L2/3. L2/3 pIPFC neurons were differentiated from L5 neurons by packing density and a change in the laminar distribution of BLA projections, where a prominent gap in BLA projections is observed between L2/3 and deep layer 5. For investigation of thalamo-cortical circuitry (Figures S4 and S6), L6 was identified by expression of strong reciprocal thalamo-cortical projections (i.e., eYFP expressing axon terminals from the MDT and rAAV positive reciprocally projecting pIPFC neurons). pIPFC neurons were voltage clamped in whole-cell configuration using borosilicate glass pipettes (2-4 MΩ) filled with internal solution containing (in mM): 125 K<sup>+</sup>-gluconate, 4 NaCl, 10 HEPES, 4 MgATP, 0.3 Na-GTP, and 10 Na-phosphocreatine (pH 7.30-7.35). For all experiments other than those shown in Figure S2, neurons were clamped at -70mV and 50  $\mu$ M picrotoxin (Cayman Chemical, Ann Arbor, Michigan) was included in the patch pipette. Following break-in to the cell, we waited  $\geq 3$  minutes to allow for exchange of internal solution and stabilization of membrane properties. Neurons with an access resistance of > 20MΩ or that exhibited greater than a 20% change in access resistance during the recording were not included in our datasets.

# Ex vivo optogenetics

For electrophysiological interrogation of the BLA-pIPFC circuit, mice were bilaterally injected with 250  $\mu$ L of AAV5-CaMKII $\alpha$ -ChR2(H134R)-eYFP and rAAV2-CAG-tdTomato in a 2:1 ratio into the BLA or MDT. 3-5 weeks of viral expression was allowed prior to sacrificing the mice.  $\geq$  1 week prior to electrophysiological analysis, littermate-matched mice were singly housed. 24 hours before electrophysiological analysis, littermate-matched mice were randomly assigned to receive either 20 randomly interspersed 0.5 mA shocks over a 10-minute period or no shocks.

For optogenetic recordings of input/output curves, we used a Thorlabs LEDD1B T-Cube driver and obtained separate recordings of 470nm wavelength oEPSCs at 200, 400, 600, 800, 1000, and 1200 mA of LED intensity. The same stimulation paradigm was used for current clamp input/output curves. Current clamp recordings of somatic current injection induced AP firing were obtained by initially injecting enough current to hold the neuron at -70mV and then applying sequential depolarizing steps that increase by 20pA. PPR recordings of oEPSCs were obtained in voltage-clamp with an inter-stimulus interval of 50ms. PPR is reported as a ratio between the amplitude of the second oEPSC divided by the first. In Figure 2, PPR values are shown across the entire range of stimulus intensities. PPR effects are not dependent on the stimulus intensity; therefore, in subsequent figures, PPR values are shown at the maximum stimulus intensity. For optogenetically elicited AP firing, three 2ms light stimulations = 0.67). Recordings of DSE were obtained following at 10 s voltage step to +30mV. A baseline of 10 oEPSCs were taken prior to the depolarizing step, and all data is plotted as an oEPSC amplitude normalized to the baseline period. A light exposure time of 2ms was used for all optogenetic experiments. For recordings of asynchronous neurotransmitter release, the CaCl2·6H20 in the ACSF was replaced with SrCl2·6H20. Asynchronous release events were analyzed in a 500ms window following optogenetic stimulation. A Clampfit template was made by selecting individual asynchronous release events and averaging them. The template was then used to analyze asynchronous neurotransmitter release.

# **Fiber Photometry**

Mice were placed on a stereotaxic frame and unilateral holes drilled over PL (AP =  $\pm 2.0$  mm, ML =  $\pm 0.3$  mm) and BLA (AP = -1.75 mm, ML =  $\pm 3.3$  mm). A 10 µL Nanofil syringe (World Precision Instruments) fitted with a 33-gauge beveled needle and connected to an infusion pump was used to microinject 200nl of AAV1.Syn.GCaMP6s.WPRE.SV40 (Penn Vector Core) into the BLA (DV = 4.6 mm) at a rate of 50 nl/min. A 400 µm diameter optical fiber (Doric) was implanted into the PL (DV = -1.6 mm) and secured to the skull with Metabond (Parkell). Two to four weeks after surgery, mice underwent the acute shock exposure protocol with concurrent recording of GCaMP6s signal. The fiber photometry rig was based on a previously described design (Cui et al., 2013; Gunaydin et al., 2014); briefly, to induce GCaMP6s fluorescence, 470 nm wavelength light emitted from an LED (Thorlabs) was passed first through a filter (Semrock, FF02-472/30) and then connected to the fiber implant with a 0.48 NA fiber optic patch cord (Doric). Activity-dependent GCaMP6s fluorescent signal was then transmitted through the fiber optic patch cord and separated from the excitation light with a dichroic (Semrock, FF495-Di03) and then passed through a single band filter (Semrock, FF01-535/50) and focused on a photodetector (Newport, Model 2151) until finally being recorded by a real-time processor (Tucker Davis Technologies). A pulse from the behavioral set-up (Video Freeze) was used to time lock fluorescent signal recordings with shock

presentations. To confirm fiber placement and GCaMP6s expression, mice were perfused, and brains were sectioned for histological verification of injection sites and fiber optic placement using Stereo Investigator software (MBF Bioscience) with a fluorescent microscope (Nikon Eclipse 80i).

# **Miniendoscopy**

For *in vivo* single cell Ca<sup>2+</sup> imaging, mice were injected with AAV expressing GCaMP7f into the pIPFC at 2 different levels (1.6 and 1.9 DV, 300 nL per injection). Following or prior to injection, a 0.5 mm diameter tract was created over PL with a blunt needle connected to a vacuum line stereotaxically driven to DV 1.55. Thereafter, a 0.5 mm diameter GRIN lens (Inscopix, Palo Alto, CA) was introduced into the tract and slowly lowered into position (1.8 DV). Animals were allowed to recover for at least 2 weeks, after which a baseplate was installed over the lens in order to dock the miniaturized microscope at an empirically optimized working distance. Mice were habituated to the microscope for at least 2 days. On test day, mice were exposed to 20 shocks as described below (**Foot-shock stress**) and the recording system (nVista, Inscopix, Palo Alto, CA) was synchronized to the fear conditioning software (FreezeFrame, actimetrics) via a TTL pulse. Data was acquired at a frame rate of 10 Hz. Laser power, gain, and lens focus were empirically adjusted to maximize the quality of the recordings. Data was acquired continuously throughout the duration of the session.

# Lipid analysis

Mice underwent cervical dislocation immediately followed by decapitation. The brain was quickly removed and placed in a brain matrix. 2 mm thick coronal sections containing the target brain regions were frozen on a metal block in dry ice. Dissections were performed on the frozen tissue using a 2 mm diameter metal micropunch. Samples were stored at -80 °C until extraction. Lipids were extracted from brain tissue by sonication in 1 mL of acetonitrile (ACN). The samples were sonicated at 60% power for 1 min while incubated in an ice bath to prevent sample heating (the sonicator was a Hielscher UP100H ultrasonic device – 100W, 30kHz). The ACN contained the following internal standards: 2-AG-d5 (1 nmol), AEA-d4 (2 pmol), AA-d8 (2 nmol), OG-d5 (0.25 nmol) and OEA-d4 (25 pmol) was included for selected samples. The sonicated homogenate was stored at -20°C overnight and then centrifuged at 4°C for 5 min at 3,000 rcf. The supernatant was dried under nitrogen. Samples were re-suspended in 200  $\mu$ L of methanol:water (50:50), followed by brief centrifugation to pellet any solid material. The cleared samples were transferred to autosampler vials and analyzed via LC-MS/MS as described in Bedse et al. (2017).

# **Foot-shock stress**

Foot-shock stress occurred 24 hours before behavioral testing and consisted of twenty unpredictable 0.1, 0.25, or 0.5 mA footshocks within 10 min using a MED Associates fear-conditioning chamber (St. Albans, VT, USA). 24 hours after foot-shock stress mice were tested in elevated zero maze or elevated plus maze test.

# **Elevated-zero maze**

The elevated-zero maze (EZM, San Diego Instruments, San Diego, California, USA) is an annular white platform and divided four equal quadrants. It consisted of two open arms and two closed arms. The outer and inner diameters of the EZM were 60.9 cm and 50.8 cm, respectively. The apparatus was elevated 60.9 cm from the floor. Light levels in the open arms were approximately 200 lux, while the closed arms were < 100 lux. Mice were placed in the closed arm of the maze and allowed to explore for 5 min. ANY-maze (Stoelting, Wood Dale, Illinois, USA) video-tracking software was used to monitor and analyze behaviors during the test.

# **Elevated-plus maze**

The elevated plus maze was custom built (Vanderbilt Machine Shop, Nashville, TN) and consists of two pairs of open and closed arms which intersect in an open center platform. The total length of each set of arms is 27," and the closed arms are bordered with 6" walls. The maze platform is elevated to 15.5." Mice were placed in the maze center, facing the entrance to the closed arm away from the experimenter. Light was set to a lux value of 150-200 for the open arms. Mice were allowed to roam for five minutes, and movement was measured with ANY-maze software (Stoelting Co., Wood Dale, IL).

# **INTRSECT** behavior

Littermate-matched homozygous CB1<sup>f/f</sup> mice were bilaterally injected with 500  $\mu$ L AAV5-CMV-fDIO-Cre-mNeonGreen into the BLA and 200  $\mu$ L rAAV2-EF1 $\alpha$ -mCherry-IRES-FIp or mCherry control virus (rAAV2-EF1 $\alpha$ -mCherry) into the pIPFC. 6 weeks after stereo-taxic surgery, mice were singly housed and allowed to acclimate for > 1 week. Prior to testing, mice were transported from a housing room to a nearby experimental room. We then allowed at least 10 minutes for mice to habituate to the experimental room. Mice were then run through EZM and Light/Dark box test (data not shown) on non-sequential days. One day following the last behavioral test, mice were exposed to the aforementioned 20 foot-shock stress protocol, and 24 hours later, run through the EPM test. For both the EZM and EPM, open arm light levels were set to 150 lux, and anxiety-like behaviors were analyzed by examining open arm entries and % open arm time using ANY-Maze software (Stoelting Co., Wood Dale, IL). The experimenter was blinded to the experimental group of the mice.

# **Chemogenetic behavior**

Littermate-matched WT C57 mice were bilaterally injected with rAAV2-pmSyn1-EBFP-Cre in the pIPFC, and AAV5-hSyn-DIO-hM3D(Gq)-mCherry or AAV5-hSyn-DIO-mCherry control virus in the BLA. A minimum of six weeks was allowed for virus expression. Thirty minutes prior to testing, mice were given I.P. administration of Clozapine N-Oxide (CNO) (MilliporeSigma, St. Louis, MO) at 5mg/kg. Mice were transported from a housing room to a nearby experimental room. We then allowed at least 20 minutes for mice to habituate to the experimental room. The EPM assay was run as described above.

# Immunohistochemistry and Imaging

Mice were anesthetized using isoflurane and transcardially perfused with ice-cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) solution in PBS. Brains were dissected and stored overnight in 4% PFA and transferred to a 30% sucrose solution for four days. 40 µm brain sections were taken using a Leica CM3050 S cryostat (Leica Microsystem, Weitzlar, Germany). Brain sections were then washed in Tris-Buffered Saline (TBS) 3X for 10 minutes. Slices were then directly mounted on glass slides and VectaShield H-1200 DAPI mounting medium (Vector Laboratories, Burlingame, CA) was applied before coverslipping. Images were taken using an Axio Imager M2 epifluorescent microscope. Whole slice images were acquired using a 5x objective while zoomed in images of the BLA or pIPFC were acquired using a 10 or 20x objective. Brightness and contrast were adjusted using Adobe CS4 software for presentation in figures.

# **cFOS** staining

Four to six weeks after intracranial viral injection surgery, both GqDREADD and mCherry mice were given an IP injection of 10mg/kg CNO-HCl dissolved in saline. Two hours after IP injection, mice were perfused as previously described. 40  $\mu$ m sections were cut, and immediately washed in TBS 3X for 10 minutes. Slices were then washed in a blocking buffer 1x TBS containing 4% horse serum and 0.2% Triton X-100 (Fisher Scientific)(TBS+). Slices were then placed into 1.5 mL Eppendorf tubes containing the blocking buffer and 1:500 Rabbit- $\alpha$ -cFOS antibody (abcam) and covered and put on a nutator overnight. The following day, slices were washed 3X in TBS+, and then put into Eppendorf tubes containing TBS+ and 1:1000 Alexa Fluor 488 Donkey- $\alpha$ -Rabbit and allowed to incubate for two hours. Slices were subsequently washed and mounted for image analysis.

### Viruses

For chemogenetic manipulation of the BLA-pIPFC circuit, we used rAAV2-Syn1-eBFP-Cre (200  $\mu$ L) in the pIPFC and AA5-hSyn-DIO-hM3d(Gq)-mCherry (500  $\mu$ L)(Addgene, Cambridge MA) in the BLA. For electrophysiological interrogation of the BLA-pIPFC circuit, we used AAV5-CaMKII-ChR2(H134R)-eYFP (UPenn Vector Core, Philadelphia, PA) and rAAV2-CAG-tdTomato (Addgene). We combined these viruses in a 2:1 ratio and injected a total volume of 250  $\mu$ L into the BLA. For pIPFC deletion of DAGL $\alpha$ , we used AAV5-CMV-Cre-eGFP (200  $\mu$ L)(Addgene). For deletion of the CB1 receptor from BLA cells projecting to the pIPFC, we designed a custom Flp recombinase dependent Cre virus, AAV5-CMV-fDIO-Cre-P2A-mNeonGreen(500  $\mu$ L into bilateral BLA)(Catalogue# VB180530-1030aad, VectorBuilder, Shenandoah, TX) and used a commercially available retrograde Flp virus, rAAV2-EF1 $\alpha$ -mCherry-IRES-Flp (200  $\mu$ L into bilateral pIPFC). For electrophysiological validation of this approach, we injected a 600  $\mu$ L combination of custom fDIO-Cre virus with AAV5-EF1 $\alpha$ -DIO-ChR2(H134R)-eYFP in a 3:1 ratio. Finally, for *in vivo* fiber photometry experiment, we used AAV1.Syn.GCaMP6s.WPRE.SV40 (Penn Vector Core) which was bilaterally injected into the BLA at a volume of 200  $\mu$ L. Also see Key Resources Table.

# **QUANTIFICATION AND STATISTICAL ANALYSIS**

# Electrophysiology

Electrophysiological data was initially analyzed using ClampFit 10.5 software (Molecular Devices, San Jose, California). Datasets were organized in Microsoft Excel and then transferred to GraphPad Prism 6.0 for generation of graphs and statistical analyses. All statistical tests are reported in the figure legends. For analysis of two groups, an unpaired Student's t test was used, with error bars indicating the mean  $\pm$  SEM. For analysis of three more groups, a one-way ANOVA with Holm-Sidak post hoc correction was used, with error bars indicating the mean  $\pm$  SEM. For analysis of two or more groups across two or more treatments or time points, a two-way ANOVA with Holm-Sidak post hoc correction was used. For all datasets, significance was defined by a p value of < 0.05. The Grubbs outlier test was run on each dataset individually and outliers were excluded from our data. Mice were excluded from physiological experiments if there was improper targeting of the BLA or pIPFC or if there was no viral expression in pIPFC terminals. Neurons were excluded from physiological experiments for four reasons. 1: if the holding current dropped below –200 pA at any time during the recording. 2: if the access resistance was > 20 M $\Omega$ . 3: if the access resistance fluctuated by more than 20% throughout the recording. 4: There was no optogenetically-evoked response. Paired-pulse ratios were only taken from neurons in which both the first and the second oEPSC had an amplitude  $\geq$  50pA.

# **Behavior**

All behavior was analyzed via ANY-maze (Stoelting, Wood Dale, Illinois, USA) software. All statistical tests are reported in the figure legends. For analysis of two groups, an unpaired Student's t test was used, with error bars indicating the mean ± SEM. For analysis of

three more groups, a one-way ANOVA with Holm-Sidak post hoc correction was used. For analysis of two or more groups across two or more treatments or time points, a two-way ANOVA with Holm-Sidak post hoc correction was used. For all datasets, significance was defined by a p value of < 0.05. The Grubbs outlier test was run on each dataset individually and outliers were excluded from our data. Mice were excluded from behavioral experiments if any of the viral injections were misplaced or if there was no viral expression. Furthermore, mice were excluded if there was a technical issue during the behavioral experiment (e.g., mouse fell out the EZM/EPM or there was a malfunction on the behavioral hardware or software).

# **cFOS** analysis

For cFOS imaging, a set exposure time of 400 ms for the GFP channel was used to ensure that there was no bias in the image acquisition process. Scale bars were included on each image to allow for subsequent processing. Raw 10x images were then opened with ImageJ, and the scale was set using the aforementioned scale bar. Images were then thresholded, and the region of interest was drawn using the ImageJ software with the Allen Brain Atlas being used to ensure the correct region was selected. ImageJ particle counting software was then used to analyze the number of cFOS cells in the region of interest. This number was then divided by the area of the region of interest to get the number of cFOS cells per square mm. Data were analyzed via an unpaired Student's t test.

# Miniendoscopy

Recordings were spatially downsampled by a factor of 2, bandpass filtered, and motion corrected using Inscopix Data Processing software V1.2. Individual  $Ca^{2+}$  traces were extracted using Constrained Nonnegative Matrix Factorization for miniendoscopic data (CNMFE) (Zhou et al., 2018). For CNMFE, we used the following parameters: min corr = 0.9, min pnr = 20, gSiz = 20, gSig = 10. For our analysis we used the raw extracted values rather than the denoised values. Individual extracted traces and corresponding identified neurons were visually confirmed and traces that corresponded to artifacts rather than neurons were excluded. For bulk fluorescence analysis, the total fluorescence for the full field of view was extracted from recordings prior to band pass filtering. The baseline and stimulus response periods were defined as the 10 s preceding shock onset and the 10 s following tone onset. For bulk fluorescence signal, the total fluorescence was averaged across the 20 stimulus response periods. Similarly, for single cell analysis, data from each individual cell was averaged across the 20 stimulus trials and then binned into 1 s intervals. Both total fluorescence signals and individual traces were quantified using custom code in MATLAB. To normalize the data, z-scores were calculated using the 10 s pre-shock period as the baseline. Traces exceeding a z-score value of 1.645 or -1.645 (p < 0.05, two-tails) for any of the following 3 1 s bins following shock onset were considered excitatory shock-responsive or inhibitory shock-responsive, respectively.

# **Fiber Photometry**

Fiber photometry data was analyzed using custom code in MATLAB, where  $\Delta$ F/F was calculated to normalize fluorescent signal data from each mouse and z-scored to account for between-subject variability in signal magnitude. To control for photobleaching, median fluorescence during a rolling window of 80 s (40 s before and 40 s after every given data point) was calculated and subtracted from each data point across the recording session.

# DATA AND CODE AVAILABILITY

The codes generated during this study are available at https://github.com/dmarcus22/NEURON-D-19-00796R2 or by request from lead contact (sachin.patel@vanderbilt.edu).