Stress-induced modulation of endocannabinoid signaling leads to delayed strengthening of synaptic connectivity in the amygdala

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Contributed by Bruce S. McEwen, October 25, 2019 (sent for review June 20, 2019; reviewed by Ron Stoop and Jeffrey G. Tasker)

Even a brief exposure to severe stress strengthens synaptic connectivity days later in the amygdala, a brain area implicated in the affective symptoms of stress-related psychiatric disorders. However, little is known about the synaptic signaling mechanisms during stress that eventually culminate in its delayed impact on the amygdala. Hence, we investigated early stress-induced changes in amygdalar synaptic signaling in order to prevent its delayed effects. Whole-cell recordings in basolateral amygdala (BLA) slices from rats revealed higher frequency of miniature excitatory postsynaptic currents (mEPSCs) immediately after 2-h immobilization stress. This was replicated by inhibition of cannabinoid receptors (CB_1R), suggesting a role for endocannabinoid (eCB) signaling. Stress also reduced *N*-arachidonoylethanolamine (AEA), an endogenous ligand of CB_1R. Since stress-induced activation of fatty acid amide hydrolase (FAAH) reduces AEA, we confirmed that oral administration of an FAAH inhibitor during stress prevents the increase in synaptic excitation in the BLA soon after stress. Although stress also caused an immediate reduction in synaptic inhibition, this was not prevented by FAAH inhibition. Strikingly, FAAH inhibition during the traumatic stressor was also effective 10 d later on the delayed manifestation of synaptic strengthening in BLA neurons, preventing both enhanced mEPSC frequency and increased dendritic spine-density. Thus, oral administration of an FAAH inhibitor during a brief stress prevents the early synaptic changes that eventually build up to hyperexcitability in the amygdala. This framework is of therapeutic relevance because of growing interest in targeting eCB signaling to prevent the gradual development of emotional symptoms and underlying amygdalar dysfunction triggered by traumatic stress.

eCB | stress | BLA | FAAH

Growing evidence points to contrasting features of stress-induced plasticity in the amygdala versus the hippocampus, both of which are essential components of the neural circuitry mediating stress responses (1). Stress-induced modulation of amygdalar structure and function is also unique in its temporal profile. For example, a single 2-h episode of immobilization triggers a delayed onset of anxiety-like behavior and spinogenesis in the basolateral amygdala (BLA) not 1 d, but 10 d later (2). Recently, electrophysiological correlates of this delayed spinogenesis have been identified—the same acute stress also enhances presynaptic glutamate release onto BLA principal neurons 10 d later (3). However, molecular signaling mechanisms underlying these delayed changes spanning both sides of the synapse, increase in postsynaptic spine density and presynaptic glutamate release, remain largely unexplored.

Several features of this rodent model of acute stress and its delayed impact are reminiscent of facets of posttraumatic stress disorder (PTSD), wherein a single, traumatic event triggers changes in affective behaviors that are both delayed and prolonged and are accompanied by amygdalar hyperactivity (4, 5). In our search for potential mechanisms underlying these delayed effects of acute stress in the BLA, we took note of reports suggesting that aberrations in endocannabinoid (eCB) signaling could play a key role in the etiology of PTSD, as well as the prevalence of cannabis use in PTSD patients to cope with their symptoms (6). The eCB system is a neuromodulatory system that not only plays a pivotal role in experience-dependent synaptic plasticity; it also links postsynaptic changes with presynaptic transmitter release (7). Specifically, this system acts through 2 postsynaptically derived ligands—*N*-arachidonylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG)—that suppress presynaptic transmitter release through the activation of cannabinoid receptors (CB_1R) localized in excitatory and inhibitory

Significance

Posttraumatic stress disorder (PTSD) is a debilitating psychiatric condition triggered by a brief traumatic event causing delayed and prolonged changes in emotional behaviors that are accompanied by amygdalar hyperactivity. Understanding of the underlying neuronal mechanisms during stress that eventually culminate in its delayed impact on the amygdala is limited. We found that oral administration, only during a brief stressor, of a pharmacological compound that elevates the levels of an endogenous ligand of cannabinoid receptors prevented both the immediate and delayed impact of acute stress on synaptic excitability in the amygdala of rats. This offers insights into potential therapeutic strategies for targeting endocannabinoid signaling to prevent the gradual development of affective symptoms and underlying amygdalar dysfunction triggered by traumatic stress.

Author contributions: F.Y., C.J.H., B.S.M., M.N.H., and S.C. designed research; F.Y., R.C., M.M., S.F., and M.N.H. performed research; M.v.d.S., Q.J.P., C.J.H., and G.C.T. contributed new reagents/analytic tools; F.Y., R.C., M.M., and M.N.H. analyzed data; and F.Y., C.J.H., B.S.M., M.N.H., and S.C. wrote the paper.

Reviewers: R.S., University of Lausanne; and J.G.T., Tulane University.

Competing interest statement: The authors declare a competing interest. This research was supported, in part, by an unrestricted operating grant to B.S.M. from Johnson & Johnson Pharmaceuticals. The funding body had no role in the design of this study, collection or analysis of the data, or decision to publish, outside of the development of the pharmacological compound, JNJ-40355003, which was used in this study.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1910322116/-/DCSupplemental.

First published December 16, 2019.

axon terminals (7). In the BLA, the eCB system, particularly AEA and the activity of its primary metabolic enzyme fatty acid amide hydrolase (FAAH), is modulated by stress (8–10). Importantly, inhibition of FAAH activity prevents the effects of chronic stress on structural plasticity in the amygdala and anxiety-like behavior in rodents (11–13).

However, these earlier findings were based primarily on the immediate effects of stress. Nothing is known about if and how eCB signaling contributes to the delayed impact of a brief stressor in the BLA. Therefore, the current study examines whether the eCB system and FAAH activity in particular play a role during acute immobilization stress that eventually leads to the delayed strengthening of both physiological and structural synaptic connectivity in the BLA days after the termination of stress.

Results

Stress Increases mEPSC Frequency in the BLA, an Effect That Is Mimicked by CB_1R Blockade. In order to assess the immediate impact of acute stress, whole-cell voltage-clamp recordings were used to measure miniature AMPAR-mediated excitatory synaptic currents (mEPSCs) from BLA principal neurons in brain slices (Fig. 1 *A* and *B*). This analysis revealed a significant enhancement in mEPSC frequency (Fig. 1 *C*, *Left*), but not amplitude (Fig. 1 *C*, *Right*), 15 min after acute stress.

As acute disruption of eCB signaling produces a plethora of neurobehavioral and endocrine responses akin to an acute stress response (14), we speculated that loss of eCB signaling may result in changes similar to what is seen following acute stress. Therefore, we determined whether blocking CB_1R in vivo results in increased mEPSC frequency in the same time frame. Ex vivo brain slices obtained 2 h after systemic administration of the CB_1R antagonist AM-251 exhibited a significant increase in mEPSC frequency without any effect on mEPSC amplitude in BLA neurons (Fig. 1 *D*–*F*).

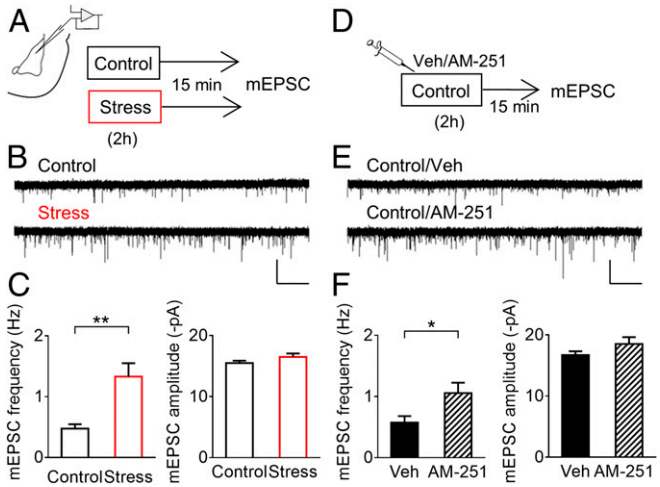

Fig. 1. Acute stress and CB_1R blockade increases mEPSC frequency in BLA principal neurons. (*A*) Schematic of part of a coronal section of the rat brain containing the BLA, showing location of recording electrode (*Left*). Experimental design depicting timeline of stress (2 h) followed by tissue collection after the termination of stress for mEPSC recordings (*Right*). (*B*) Representative mEPSC traces from control and stressed animals. (Scale bar, 30 pA, 5 s.) (*C*) After 2 h of acute stress, BLA neurons from the stressed group exhibited a significant increase (control: $n = 12$, stress: $n = 12$, $t[13.29] = 3.701$, $P = 0.0026$, $^{**}P < 0.01$) in mEPSC frequency (*Left*), but not mEPSC amplitude (*Right*). (*D*) Experimental design depicting i.p. injection of either vehicle (Veh) or AM-251 in controls, followed by mEPSC recordings. (*E*) Representative mEPSC traces. (Scale bar, 30 pA, 5 s.) (*F*) BLA neurons from the AM-251–treated group exhibited a significant increase (vehicle: $n = 11$, AM-251: $n = 15$, $t[22.06] = 2.411$, $P = 0.0247$, $^{*}P < 0.05$) in mEPSC frequency (*Left*). mEPSC amplitude was not affected by AM-251 (*Right*).

While AEA is believed to mediate tonic eCB signaling in the BLA (14), there are currently no available tools to inhibit AEA synthesis and verify this. Instead, we capitalized on the recently developed DAGL inhibitor DO34, which effectively quenches 2-AG signaling to determine if 2-AG contributes to tonic regulation of glutamatergic transmission in the BLA (15). Unlike what was seen with direct CB1 receptor blockade, no statistically significant difference was observed in mEPSC frequency and amplitude preincubated and perfused with DO34 compared to vehicle (*SI Appendix*, Fig. S1 *A* and *B*). In addition, no effect of DO34 was observed on frequency and amplitude of miniature inhibitory postsynaptic currents (mIPSCs) recorded from BLA principal neurons (*SI Appendix*, Fig. S1*C*). Together, these results suggest that tonic endocannabinoid tone in the BLA is not mediated by 2-AG and support the hypothesis that it is driven by AEA signaling.

Characterization of FAAH Inhibition in the BLA. Given that the current data suggest that AEA, not 2-AG, represents the primary "tonic" signaling molecule of the eCB system in the BLA, we explored whether acute stress-induced loss of AEA signaling in the BLA contributes to the increase in mEPSC frequency reported above. AEA concentrations were increased by pharmacologically inhibiting FAAH, the enzyme that degrades AEA and shows an increase in activity with stress (8). A noninvasive and nonstressful means of administering FAAH inhibitors was utilized in which 50 mg/kg/day of FAAH inhibitor (FAAH-I) was incorporated into the food (see *Materials and Methods*). This treatment approach did not affect food consumption. Animals treated with FAAH-I exhibited decreased maximal velocity of AEA hydrolysis by FAAH (Fig. 2 *A* and *B*). BLA AEA (Fig. 2*C*), but not 2-AG (Fig. 2*D*), concentrations were significantly elevated by FAAH-I treatment.

Stress Reduces AEA Concentrations and FAAH-I Reverses Stress-Induced Increase in mEPSC Frequency in BLA. Consistent with previous studies examining other stressors (8–10), acute immobilization stress resulted in a significant reduction in the BLA AEA content (Fig. 3 *A*, *Left* and *C*). mEPSCs were recorded from BLA neurons following FAAH-I treatment in control and stressed rats (Fig. 3 *A*, *Right*). Consistent with our initial studies, stress exposure in vehicle-treated animals resulted in higher mEPSC frequency compared to vehicle-treated control animals (Fig. 3 *B*, *Top*). The stress-induced enhancement in mEPSC frequency did not occur in animals treated with FAAH-I before stress; mEPSC frequency in FAAH-I–treated, stressed rats was comparable to frequency in control-vehicle animals and significantly less than stress-vehicle animals (Fig. 3 *B*, *Bottom* and Fig. 3 *D*, *Left*). FAAH-I administration alone in control animals did not cause any change in mEPSC frequency. mEPSC amplitude was not affected by either stress or FAAH-I administration (Fig. 3 *D*, *Right*).

Reversal of Stress Effects Caused by FAAH-I Is Prevented by Systemic Administration of AM-251 during Stress. Finally, to determine if this effect of FAAH inhibition was mediated by AEA signaling at CB_1R, rats were simultaneously treated with both FAAH-I and AM-251 during acute stress (Fig. 4*A*). AM-251 was injected i.p. immediately at the onset of stress in the immobilization bag, while FAAH-I was delivered through food as described earlier. mEPSC frequency in FAAH-I–treated animals receiving AM-251 during stress was significantly different from FAAH-I–treated animals receiving vehicle injection during stress (Fig. 4 *B* and *C*, *Left*), indicating that inhibition of CB_1R reverses the protective role of FAAH-I against stress-induced increase in glutamatergic transmission. Importantly, mEPSC frequency in the FAAH-I/AM-251 group did not differ from mEPSC frequency

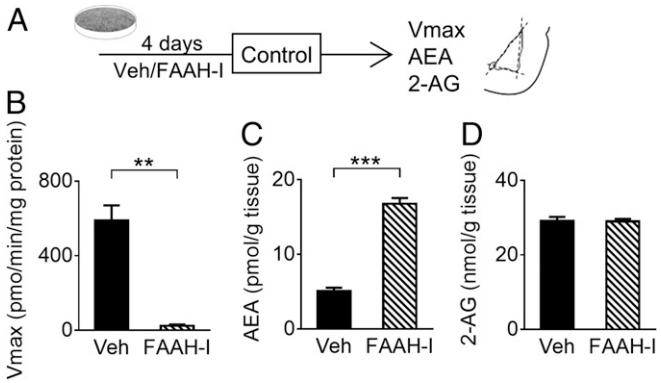

Fig. 2. FAAH inhibitor characterization. (*A*) Experimental design depicting timeline of FAAH-I administration followed by tissue collection. (*B*) Systemic administration of the FAAH-I significantly decreases the maximal velocity (V_{max}) of AEA hydrolysis by FAAH (vehicle [Veh]: $n = 4$, FAAH-I: $n = 4$, $t[3.055] = 6.963$, $P = 0.0057$, $^{**}P < 0.01$). (*C*) AEA content within the BLA significantly increases after FAAH-I treatment (vehicle: $n = 4$, FAAH-I: $n = 4$, $t[4.652] = 13.04$, $^{***}P < 0.0001$). (*D*) FAAH-I does not alter 2-AG levels in the BLA (vehicle: $n = 4$, FAAH-I: $n = 4$, $t[5.094] = 0.1210$, $P = 0.9084$).

recorded in stressed animals, providing additional support for the reversal of the effect of the FAAH-I by AM-251. In addition, antagonism of CB_1R during stress alone did not elevate mEPSC frequency (*SI Appendix*, Fig. S2). Mean mEPSC amplitudes were not different between the 2 groups (Fig. 4 *C*, *Right*).

FAAH-I Does Not Change Stress-Induced Decrease in mIPSC Frequency in BLA Neurons. CB_1R also modulates synaptic inhibition in the amygdala (16, 17); hence, we examined the impact of FAAH inhibition on stress-induced alterations in GABAergic synaptic transmission. $GABA_A$-mIPSCs in BLA neurons taken from control and stressed animals that were treated with vehicle or FAAH-I (Fig. 5*A*). In contrast to what was seen with mEPSCs, acute stress in vehicle-treated animals resulted in a reduction in mIPSC frequency compared to vehicle-treated control animals (Fig. 5 *B*, *Top* and Fig. 5 *C*, *Left*). Strikingly, FAAH inhibition had no effect on the stress-induced decrease in mIPSC frequency (Fig. 5 *B*, *Bottom* and Fig. 5 *C*, *Left*). No difference was observed in mIPSC amplitudes across the groups (Fig. 5 *C*, *Right*). Hence, FAAH and AEA do not regulate stress-induced changes in synaptic inhibition in the BLA.

Stress Enhances 2-AG and Reduces mIPSC Frequency. Two hours of acute immobilization stress produced a significant increase in 2-AG content within the BLA (Fig. 6 *A*, *Left* and Fig. 6*B*). This is consistent with recent electrophysiological findings suggesting that stress-induced release of 2-AG within the BLA suppresses GABA release and promotes anxiety (18). Hence, we reasoned that stress-induced increase of 2-AG would result in decreased inhibitory drive, and CB_1R blockade would eliminate this alteration. mIPSCs were compared in BLA taken from vehicle- and AM-251–treated, control and stressed rats (Fig. 6 *A*, *Right*). BLA neurons from stressed animals exhibited a significant decrease in mIPSC frequency compared to controls (Fig. 6 *C*, *Top* and Fig. 6*D*). Rats treated with AM-251 during stress had mIPSC frequency levels comparable to both vehicle-treated and AM-251–treated control animals (Fig. 6 *C*, *Bottom* and Fig. 6 *D*, *Left*). Thus, blockade of CB_1R using AM-251, while having no impact on basal mIPSC frequency, prevented stress-induced reduction in mIPSC frequency in the BLA. Consistent with previous reports (18), these data suggest that stress-induced release of 2-AG reduces synaptic inhibition in the BLA.

FAAH-I Prevents the Delayed Increase in mEPSC Frequency and Spine Density in the BLA 10 d after Acute Stress. The acute immobilization stress protocol used here has previously been shown to trigger a delayed increase in dendritic spine-density and excitatory synaptic currents in the BLA 10 d later (3). Given that inhibition of FAAH reduced the increased glutamate release onto BLA pyramidal neurons in response to stress, we hypothesized that FAAH inhibition would prevent the delayed synaptic effects of acute stress on BLA neurons 10 d later. Rats were administered FAAH-I or vehicle for 4 consecutive days before exposure to 2 h of immobilization stress. mEPSC recordings from BLA neurons (Fig. 7*A*) in brain slices prepared from these animals revealed stress-induced increase in mEPSC frequency in vehicle-treated rats 10 d later (Fig. 7 *B*, *Top* and Fig. 7 *C*, *Left*), which is consistent with earlier reports (3). In contrast, FAAH-I treatment before the acute stress prevented the delayed increase in mEPSC frequency; FAAH-I did not affect mEPSCs in control rats (Fig. 7 *B*, *Bottom* and Fig. 7 *C*, *Left*). mEPSC amplitudes did not differ between the 4 groups (Fig. 7 *C*, *Right*). As our current data indicate that stress reduces AEA signaling to facilitate excitatory transmission in the BLA and that disruption of tonic AEA/CB1 receptor signaling via AM-251 administration recapitulates the effects of stress, we also examined if there were protracted effects of AM-251 on mEPSC in the BLA similar to what was seen following stress. As was seen following acute stress exposure, AM-251 was also found to result in increased mEPSC frequency 10 d later (*SI Appendix*, Fig. S3). This demonstrates that a loss of tonic AEA/CB1 receptor signaling in the BLA is not only sufficient to produce acute elevations in excitatory transmission in the BLA, as does stress, but also results in sustained changes in BLA excitability.

Finally, to determine if the stress-induced changes in mEPSC that were attenuated by FAAH inhibition also related to anatomical changes in BLA neurons, we examined the effects of FAAH-I administration on stress-induced increase in spine-density in BLA principal neuron 10 d after acute stress. Spine numbers on

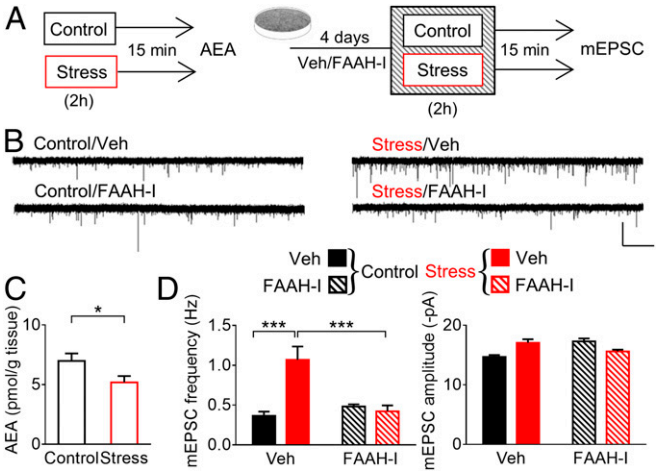

Fig. 3. Acute stress reduces AEA, and FAAH inhibitor blocks acute stress-induced immediate increase in mEPSC frequency in BLA neurons. (*A*) Experimental design depicting timeline of stress (2 h) followed by tissue collection (*Left*) and timeline of FAAH-I administration followed by mEPSC recordings after stress (*Right*). (*B*) Representative mEPSC traces. (Scale bar, 30 pA, 5 s.) (*C*) Acute stress results in a significant reduction in tissue content of AEA within the amygdala (control, $n = 12$; stress, $n = 12$; $t(21.36) = 2.195$; $P = 0.0390$, $^{*}P < 0.05$). (*D*) Vehicle (Veh)-treated stressed group ($n = 13$) has a significantly higher mEPSC frequency compared to vehicle-treated controls ($n = 14$). FAAH-I before stress ($n = 13$) led to reduced mEPSC frequency. FAAH-I in controls ($n = 13$) did not cause change in mEPSC frequency. (Interaction, $F[1,49] = 16.12$; $P = 0.0002$, $^{***}P < 0.001$; *Left*). Summary of average mEPSC amplitudes (*Right*).

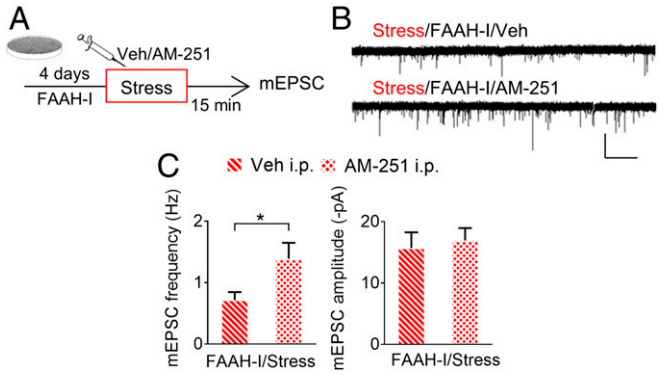

Fig. 4. Rescue of mEPSC frequency by FAAH inhibitor in BLA neurons is prevented by blocking CB_1R during stress. (*A*) Experimental design depicting timeline of FAAH-I administration followed by either vehicle (Veh) or AM-251 i.p. injection during stress. (*B*) Representative mEPSC traces. (Scale bar, 20 pA, 3 s.) (*C*) mEPSC frequency (*Left*) in the FAAH-I–treated group ($n = 13$) which received an i.p. injection of AM-251 is significantly different (Mann–Whitney *U* test, $U = 25$, $P = 0.025$, $^*P < 0.05$) from the FAAH-I–treated group which receive vehicle injection ($n = 9$) during stress. Summary of mEPSC amplitudes (*Right*).

primary dendritic branches of Golgi-stained BLA principal neurons were quantified (Fig. 7*D*). Consistent with earlier studies, stress exposure caused a significant increase in the number of spines in vehicle-treated rats 10 d later (2, 3). However, FAAH-I treatment prevented this delayed increase in BLA spine-density in stressed rats (Fig. 7 *E*, *Left*). We followed this up with a detailed analysis of spine density along 10-μm segments of dendrite. This showed how the prevention of delayed spinogenesis by FAAH-I is distributed across primary apical dendrites (Fig. 7 *E*, *Right*). Together, these results establish how inhibition of FAAH during acute stress not only prevents its delayed impact on enhanced mEPSC frequency, but also blocks the delayed increase in spine density 10 d later.

Discussion

Here we explored the mechanism underlying how a single exposure to 2 h immobilization stress culminates 10 d later in synaptic changes in the BLA that include increased synaptic excitability and new spines. Using electrophysiological analyses in ex vivo BLA slices obtained 15 min after the end of stress, our results demonstrate enhanced mEPSC frequency. In vivo inhibition of CB_1Rs replicated this increase in excitatory glutamatergic transmission, suggesting that tonic eCB signaling is involved in the maintenance of glutamatergic transmission in the BLA. This effect was not recapitulated following local depletion of 2-AG, suggesting that AEA likely mediates tonic control of glutamatergic transmission in the BLA. To further explore the nature of this relationship under conditions of stress, we probed the regulatory role of FAAH, the enzyme that hydrolyzes the endogenous CB_1R ligand AEA and whose activity increases after acute stress (8, 10). We found that noninvasive, oral administration of an FAAH-I prevented the increase in mEPSC frequency in BLA slices immediately after stress. FAAH-I treatment prior to stress prevented both the delayed morphological (increase in the number of dendritic spines) and electrophysiological strengthening (higher mEPSC frequency) of BLA synapses caused by acute stress. This is consistent with previous reports that ablation of FAAH can prevent dendritic structural plasticity produced by exposure to chronic stress or corticosterone (11, 12).

What are the potential mechanisms involving modulation of eCB signaling and synaptic transmission in the BLA that enable inhibition of FAAH activity to prevent the impact of stress on BLA synaptic connectivity? While in a previous study we reported that the same acute stress enhanced presynaptic release of glutamate, as evidenced by higher mEPSC frequency 10 d later (3), we now show that it also increases mEPSC frequency immediately after stress. This adds to accumulating evidence on enhanced glutamatergic synaptic transmission in the BLA following other stressors (19–21). Numerous reports have suggested that tonic or constitutive eCB/CB_1R signaling constrains activation of stress responsive systems in the brain at rest and that disruption of this signal can produce neural, endocrine, and behavioral responses akin to what is seen following acute stress. Specifically, acute disruption of eCB signaling increases activation of the hypothalamic–pituitary–adrenal axis, induces c-fos expression in neural circuits mediating the stress response, increases behavioral indices of anxiety, and impairs fear extinction (9, 14, 22–24). Here we provide direct physiological evidence that pharmacological inhibition of eCB signaling also increases glutamatergic transmission, similar to acute stress.

These findings suggest a model in which increased activation of the enzyme FAAH reduces AEA content following exposure to stress, which facilitates excitatory synaptic transmission by relieving tonic eCB-mediated suppression of synaptic glutamate release. Our group has previously demonstrated that corticotropin-releasing hormone (CRH) mediates the stress-induced increase in FAAH (11), and it has also been reported that corticosterone can also facilitate glutamate release in the BLA via a presynaptic mineralocorticoid receptor (MR)-mediated mechanism (20). As such, these data would suggest that both stress-mediators CRH and corticosterone may coordinately increase glutamate release in the BLA on differing time scales, with CRH perhaps rapidly producing these changes through dynamic effects on AEA signaling, as is demonstrated in the current paper, while progressive increases in corticosterone following stress enhance this process via MR in the BLA.

Consistent with earlier reports (18, 25), exposure to stress reduced mIPSCs, but this was not affected by FAAH inhibition. Interestingly, the suppression of GABA release was partially reversed by the CB_1R antagonist AM-251 and was accompanied by increased 2-AG content in the BLA, suggesting that reduced

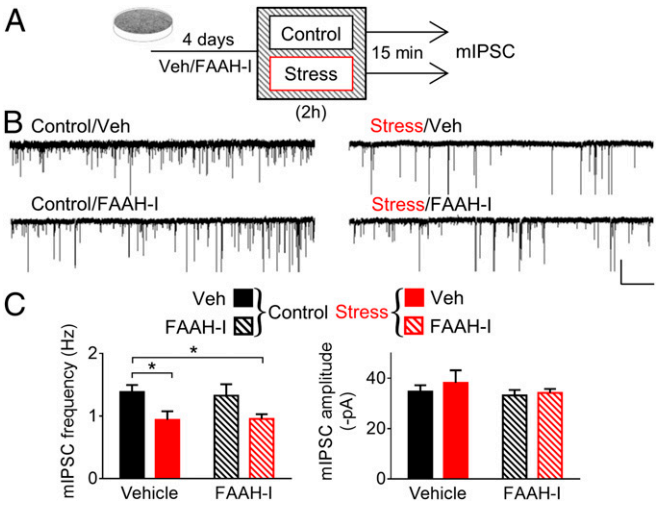

Fig. 5. FAAH inhibitor does not change acute stress-induced immediate decrease in mIPSC frequency in BLA neurons. (*A*) Experimental design. (*B*) Representative mIPSC traces. (Scale bar, 50 pA, 5 s.) (*C*) Vehicle (Veh)-treated stressed group ($n = 14$) has lower mIPSC frequency compared to vehicle-treated controls ($n = 12$). FAAH-I before stress ($n = 12$) also reduced mIPSC frequency compared to FAAH-I treatment in controls ($n = 13$) as well as vehicle-treated controls (stress: $F[1,47] = 9.18$, $P = 0.004$; drug: $F[1,47] = 0.03$, $P = 0.87$; interaction: $F[1,47] = 0.07$, $P = 0.79$), $^*P < 0.05$) (*Left*). Summary of average mIPSC amplitudes illustrates no difference between the groups (*Right*).

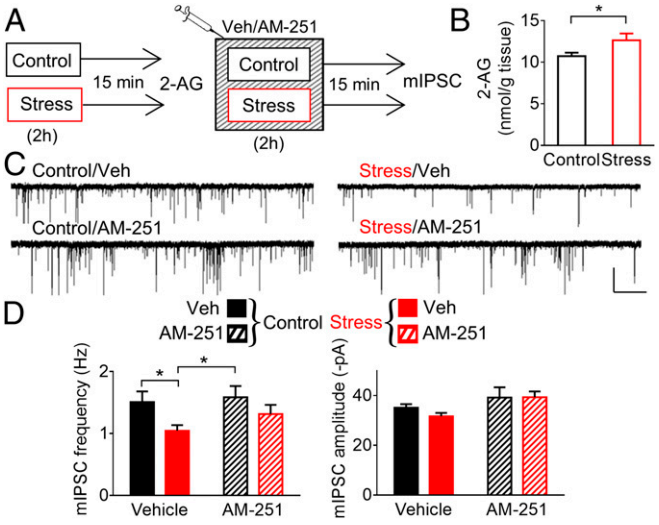

Fig. 6. Acute stress enhances 2-AG and results in CB_1R-mediated reduction in mIPSC frequency in BLA principal neurons. (*A*) Experimental design depicting timeline of stress (2 h) followed by tissue collection (*Left*) and stress followed by immediate i.p. injection of either vehicle (Veh) or AM-251, in addition to their respective controls (*Right*) for mIPSC recordings. (*B*) Acute stress results in a significant enhancement of 2-AG within the amygdala (control: $n = 12$, stress: $n = 12$, t [22] = 2.094, $P = 0.048$, $^*P < 0.05$). (*C*) Representative mIPSC traces. (Scale bar, 50 pA, 5 s.) (*D*) Vehicle-treated stressed group ($n = 15$) has significantly lower mIPSC frequency compared to vehicle-treated controls ($n = 14$). Group treated with AM-251 during stress ($n = 11$) has mIPSC frequency comparable to AM-251–treated controls ($n = 15$). (Stress: F[1,51] = 5.58, $P = 0.02$; drug: F[1,51] = 1.26, $P = 0.27$; interaction: F[1,51] = 0.38, $P = 0.54$, $^*P < 0.05$ (*Left*). Summary of average mIPSC amplitudes (drug: F[1,51] = 4.84, $P = 0.03$) (*Right*).

inhibitory tone in the BLA in response to stress was mediated, in part, by an increase in 2-AG signaling, which is consistent with previous reports (18). This ability of AEA to preferentially target glutamatergic CB1 receptors, while 2-AG may have a greater influence on GABAergic CB1 receptors, is consistent with previous physiological, neurochemical, and behavioral reports and may have implications for drugs targeting FAAH versus monoacylglycerol lipase for the potential treatment of stress-related disorders given the differential effects these approaches may have on excitability of the BLA, as well as the generation of fear and anxiety (26, 27).

The loss of synaptic inhibition following stress, coupled to the higher levels of synaptic glutamate in the BLA during and soon after stress, is likely to act to enhance the excitability of BLA pyramidal neurons. Thus, our working model is that elevations in AEA/CB_1R signaling following FAAH inhibition counter the delayed effects of stress on the BLA through an attenuation of afferent excitatory drive. With respect to potential mechanisms that could translate the enhanced release of glutamate triggered immediately after acute stress into long-term synaptic changes that are eventually evident 10 d later, we have recently identified a role for *N*-methyl-D-aspartate (NMDA) receptors in the BLA during acute stress in the delayed formation of dendritic spines (3). This raises the possibility that enhanced glutamate levels, by activating NMDA receptors in the BLA, could lead to an ideal synaptic substrate for spinogenesis. Such a scenario would be consistent with accumulating evidence that long-term potentiation leads to creation of new dendritic protrusions and spines (28, 29). Additionally, long-term potentiation is known to depend on NMDA receptors, both of which are enhanced in the BLA following stress. Finally, brain-derived neurotrophic factor (BDNF) is a potent regulator of morphological plasticity of dendritic spines in various brain regions. The same acute stress used here is known to cause a robust increase in BDNF levels in the BLA (30). Specifically, the highest levels of BDNF in the BLA were seen 1 d after acute stress, and 10 d later, the BLA continued to express significantly elevated levels of BDNF (30). Collectively, these mechanisms could mediate the progressive increase in spines on BLA pyramidal neurons following stress. As such, AEA could influence these delayed changes by its ability to acutely dampen stress-induced glutamate release.

From a translational perspective, this model may also help to explain how genetic variance in the FAAH gene can modulate anxiety and activation of the amygdala in response to stress. In humans, there is a single-nucleotide polymorphism (P129T FAAH SNP; rs324420) that exists within the FAAH gene that results in a consequential reduction in FAAH expression and a moderate facilitation of AEA signaling (31). Interestingly, humans bearing

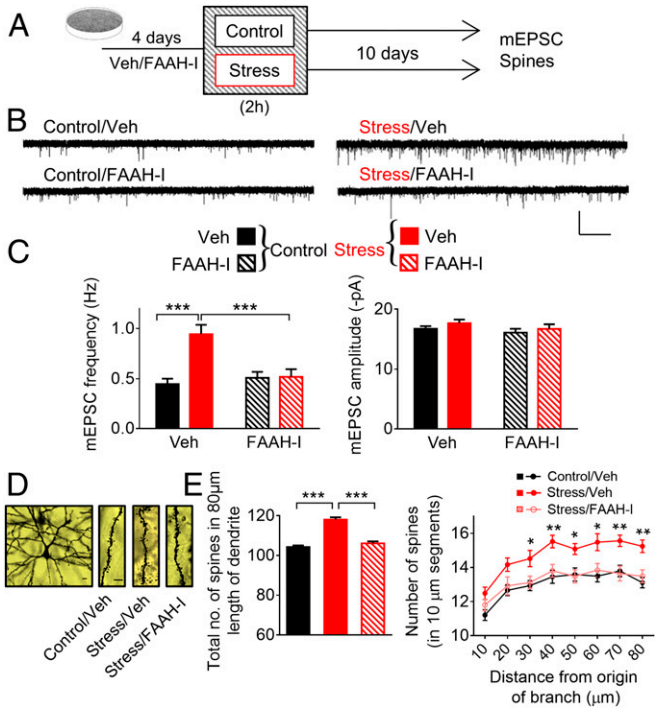

Fig. 7. Stress-induced delayed increase in mEPSC frequency and spine density in BLA principal neurons is blocked by FAAH-I. (*A*) Experimental design depicting timeline of FAAH-I administration followed by stress, 10 d after which either mEPSC recordings were done or brains were processed for spine analysis. (*B*) Representative mEPSC traces. (Scale bar, 30 pA, 5 s.) (*C*) Vehicle (Veh)-treated stressed group has increase in mEPSC frequency 10 d after stress. This delayed increase is not observed in the FAAH-I–treated stressed group (control/vehicle: $n = 16$, stress/vehicle: $n = 16$, stress/FAAH-I: $n = 16$, control/FAAH-I: $n = 16$; stress: F[1,60] = 11.79, $P = 0.001$; drug: F[1,60]= 6.04, $P = 0.02$; interaction: F[1,60] = 10.91, $P = 0.002$, $^{***}P < 0.001$ (*Left*). Summary of average mEPSC amplitudes shows no difference between the 4 groups (*Right*). (*D*) Low-power photomicrograph of a Golgi stain-impregnated pyramidal neuron in the BLA (20×; Scale bar, 10 µm) (*Left*). Representative images of primary dendrites of BLA pyramidal neurons (40×; Scale bar, 10 µm) (*Right*). (*E*) Analysis of total number of spines on a primary branch of pyramidal neurons in the BLA shows an increase in spine density in the vehicle-treated stressed group ($n = 25$) compared to vehicle-treated controls ($n = 29$). FAAH-I treatment ($n = 25$) rescues stress-induced increase in spines in the BLA (one-way ANOVA, F[2,76] = 58.17, $^{***}P < 0.001$) (*Left*). Segmental analysis of dendritic spine density in each successive 10-µ segment along a primary branch in the pyramidal neurons in the BLA as a function of distance of that segment from the origin of the branch. Significant differences observed are indicated (for stress/vehicle vs. stress/FAAH-I) as follows: $^*P < 0.05$, $^{**}P < 0.01$ (repeated measures two-way ANOVA, Tukey's multiple comparisons test) (*Right*).

the SNP have been found to exhibit blunted activation of the amygdala in response to stressful stimuli, reduced responses to stress, accelerated fear extinction, and lower indices of anxiety. Consistent with our data, it is likely that the protective effect of the P129T FAAH SNP may relate to its ability to prevent stress-induced loss of AEA signaling within the amygdala (32), which would then restrict activation of the BLA to limit the magnitude of a stress response.

Hyperactivity of the amygdala is a hallmark finding in PTSD and is believed to relate to excess anxiety and high levels of arousal seen in the disorder (33). The formation of new spines on amygdala neurons from stress exposure or genetic mutation has been linked to enhanced fear and anxiety-like behavior in rodents (1, 2, 11). Thus, our rodent model of stress is attractive in relation to PTSD because it leads to a progressive development of anxiety that is manifested well after the acute stressor and is accompanied by cellular changes in brain structure implicated in the emotional symptoms of PTSD (2, 6). As such, the current data suggest that inhibition of FAAH could reduce hyperactivity of the amygdala and thus potentially act as a novel treatment approach for PTSD. However, FAAH inhibitor administration via the chow does not specifically rule out the possibility that the blockade could be occurring outside the BLA as well. Therefore, whether targeted blockage of FAAH in the BLA alone will be sufficient for alleviating the effects of stress is an important topic for future investigations.

Finally, while this study tells us that intervention during stress prevents its delayed effects, it also offers a framework to explore therapeutically relevant questions about interventions after stress. Specifically, future studies will be required to examine time-windows after the termination of stress wherein FAAH inhibitors would still be effective in blocking the delayed strengthening of synaptic connectivity in the BLA. In addition, use of a noninvasive, oral administration of an FAAH inhibitor highlights its therapeutic relevance. Such studies are likely to give rise to new experimental paradigms that will help us better understand and intervene against molecular and synaptic mechanisms linking traumatic stress to its eventual manifestation as debilitating psychiatric conditions.

Materials and Methods

Animals, stress, electrophysiology, staining, drug administration, endocannabinoid extraction, FAAH activity assay, and statistical analyses are described in *SI Appendix*. The Institutional Animal Ethics Committee at the Rockefeller University, University of Calgary, and National Centre for Biological Sciences approved all procedures related to animal maintenance and experimentation.

Chemicals. AM-251 was obtained from Tocris Bioscience, TTX and QX-314 from Alomone Labs. FAAH-I JNJ-40355003 was provided courtesy of Johnson & Johnson pharmaceuticals (34). All other drugs were from Sigma-Aldrich.

Data availability. All data discussed in the paper are available in *SI Appendix* and Datasets S1–S10.

ACKNOWLEDGMENTS. This work was funded by the Department of Biotechnology and the Department of Atomic Energy, Government of India (S.C.); the Department of Atomic Energy, Government of India (F.Y.); NIH Grant MH41256 (to B.S.M.); an unrestricted grant from Johnson & Johnson (to B.S.M and S.F.); a Research and Education Component of the Advancing a Healthier Wisconsin Endowment at the Medical College of Wisconsin (C.J.H.); operating fund support from the Natural Sciences and Engineering Research Council of Canada (RGPIN 05791-2014 to Q.J.P.); Canadian Institutes of Health Research (CIHR) grants (Grant FDN 143329 [to M.N.H.] and MOP 130495 [to G.C.T.]); and a postdoctoral fellowship from CIHR and Alberta Innovates Health Solutions (to M.M.). S.C. thanks Purabi Biswas for her encouragement and support.

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