Published in final edited form as:

Biol Psychiatry. 2017 October 01; 82(7): 488–499. doi:10.1016/j.biopsych.2017.03.002.

Functional Redundancy Between Canonical Endocannabinoid Signaling Systems in the Modulation of Anxiety

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Abstract

Background—Increasing the available repertoire of effective treatments for mood and anxiety disorders represents a critical unmet need. Pharmacological augmentation of endogenous cannabinoid (eCB) signaling has been suggested to represent a novel approach to the treatment of anxiety disorders; however, the functional interactions between two canonical eCB pathways mediated via anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in the regulation of anxiety are not well understood.

Methods—We utilized pharmacological augmentation and depletion combined with behavioral and electrophysiological approaches to probe the role of 2-AG signaling in the modulation of stress-induced anxiety, and the functional redundancy between AEA and 2-AG signaling in the modulation of anxiety-like behaviors in mice.

Results—Selective 2-AG augmentation reduced anxiety in the light-dark box assay, and prevented stress-induced increases in anxiety associated with limbic AEA deficiency. In contrast, acute 2-AG depletion increased anxiety-like behaviors, which was normalized by selective pharmacological augmentation of AEA signaling and via direct CB1 receptor stimulation with tetrahydrocannabinol (THC). Electrophysiological studies revealed 2-AG modulation of amygdala

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All other authors report no biomedical financial interests or potential conflicts of interest.

glutamatergic transmission as a key synaptic correlate of the anxiolytic effects of 2-AG augmentation.

Conclusions—Although AEA and 2-AG likely subserve distinct physiological roles, a pharmacological and functional redundancy between these canonical eCB signaling pathways exists in the modulation of anxiety-like behaviors. These data support development of eCB-based treatment approaches for mood and anxiety disorders and suggest a potentially wider therapeutic overlap between AEA and 2-AG augmentation approaches than previously appreciated.

Keywords

2-arachidonoylglycerol; MAGL inhibition; JZL184; anxiety; stress; amygdala

INTRODUCTION

The mood and anxiety disorders as a group are the most prevalent mental health condition (1). Between 1990 and 2013, the number of people suffering from anxiety and/or depression increased by nearly 50%, from 416 million to 615 million (2). The prevalence of mood and anxiety disorders including posttraumatic stress disorder (PTSD), panic disorder, and generalized anxiety disorders underscores the critical need for innovative treatment approaches for this class of disorders. The current primary treatment for these disorders produces a delayed onset of action with modest therapeutic benefits and significant adverse effects (3), while the chronic use of the fast-acting benzodiazepines treatment can cause potentially significant adverse effects (4–6).

The endogenous cannabinoid (eCB) system is a retrograde lipid signaling system that is implicated in the regulation of multiple physiological functions in the nervous system (7, 8). Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are two well-studied eCBs that exert biological effects via activation of type 1 and 2 cannabinoid receptors (CB1 and CB2) (9, 10). Preclinical studies support the role of the eCB system as a modulator of unconditioned anxiety-related behaviors, depressive-like behaviors, and extinction of fear memories (11–15). Although significant literature supports preclinical efficacy of AEA augmentation to treat anxiety-related disorders (11, 14, 16–23), the therapeutic potential of 2-AG augmentation is only beginning to be elucidated. 2-AG is the most abundant eCB in the brain and clinical studies have demonstrated reduced peripheral 2-AG levels in patients with PTSD and with major depression (24, 25). Consistent with these findings, we and others recently showed that genetic deficiency in 2-AG signaling is sufficient to induce anxious and depressive-like behavioral states (26, 27). Similarly, recent studies have demonstrated that augmenting 2-AG signaling can reduce anxiety-like behaviors (28–30). Despite these data a central question remains as to whether these two canonical eCB signaling systems, AEA and 2-AG, exhibit redundancy in their effect on anxiety-like behaviors, and if anxiety-related phenotypes associated with deficiencies in one system can be compensated for by augmentation of the other.

eCB signaling has also been heavily implicated in the modulation of stress response physiology and subsequent behavioral responses used to model specific dimensions of mood and anxiety disorders. Studies have generally shown that stress evokes bidirectional changes

in these two eCB molecules. For example, stress exposure reduces amygdala AEA levels, and reductions in AEA levels after stress correlate with anxiety-like behavioral states (16, 31). In contrast to AEA, stress generally increases 2-AG levels (32), which contributes to termination and adaptation to stress, as well as potentially contributing to changes in pain perception and emotional memory (13, 33).

Here we show that acute restraint stress causes a selective reduction in amygdala AEA levels and a robust anxiety-like behavioral state, which can be prevented by both AEA normalization *and* 2-AG augmentation. Moreover, acute 2-AG depletion causes an increase in anxiety-like behavior which can be reversed by AEA augmentation. Physiological studies revealed amygdala glutamatergic transmission as a key predictor of anxiety-like state and anxiolytic response to 2-AG augmentation after stress. These studies support a functional reciprocal redundancy between two canonical eCB signaling systems in the modulation of anxiety-like behaviors.

MATERIALS AND METHODS

Subjects

Male ICR (CD-1) mice between 6–9 weeks of age were used for all experiments (Envigo, Indianapolis, IN, USA). All mice were group housed on a 12:12 light-dark cycle (lights on at 6:00 a.m.) with food and water available ad libitum. All studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the Vanderbilt University Institutional Animal Care and Use Committee (#M/15/105). All behavioral testing was performed between 6:00 am and 6:00 pm.

Drugs and treatment

The drugs used were MAGL inhibitor JZL184 (3, 5, 10 and 15 mg kg⁻¹), FAAH inhibitor PF-3845 (1 mg kg⁻¹), CB1 agonist CP55940 (0.001, 0.01, 0.1 and 0.3 mg kg⁻¹), CB1 agonist THC (0.3 mg/kg⁻¹), CB1 antagonist Rimonabant (2 mg kg⁻¹) and DAGL inhibitor DO34 (50 mg kg⁻¹). JZL184, PF-3845, Rimonabant or vehicles were administered by intraperitoneal injection at a volume of 1 ml kg⁻¹ in DMSO. DO34, CP55940, DO34+PF3845, THC or vehicle were administered by intraperitoneal injection at a volume of 10 ml kg⁻¹ in a formulation containing ethanol: koliphor: saline (1:1:18). Drug pretreatment times were two hours prior to behavioral testing except THC (30 min). Diazepam was administered by intraperitoneal injection 45 min prior to behavioral testing.

Supplementary Methods

For a detailed description of stress exposure, light-dark box test, lipid analysis, ex vivo slice electrophysiological recordings and statistics, see supplementary materials.

RESULTS

MAGL inhibition reduces anxiety-like behavior

To elucidate the role for 2-AG signaling in the regulation of anxiety we utilized the MAGL inhibitor JZL184. JZL184 administered at 3, 5, 10 or 15 mg kg⁻¹ elevated 2-AG levels by 18, 41, 71 and 135 %, respectively (Fig. 1A). In line with previous studies, elevations in brain 2-AG were accompanied by significant reductions in the levels of arachidonic acid (AA) and N-arachidonoylglycine (NAGly) (Fig. 1B–C) (34). As expected, 2-OG levels were increased dose-dependently (Fig. 1D). Importantly, brain AEA levels were unaffected by JZL184 at all tested doses (Fig. 1E). Other N-acyl ethanolamines (NAEs), such as N-oleoylethanolamine (OEA) and palmitoylethanolamine were likewise unaffected at any dose of JZL184 tested (OEA, Fig. 1F and PEA, supplementary Fig. S1A). Plasma 2-AG, 2-OG, but not AEA, were also increased in a dose-dependent manner by JZL184 treatment (supplementary Fig. S1B–F). JZL184 was detected at high levels in brain and plasma after i.p. injection (Fig. 1G–H), and plasma JZL184 levels were positively correlated with brain JZL184 levels (Fig. 1I) and 2-AG levels (Fig. 1J), but not with AEA levels (Fig. 1K). Moreover, plasma and brain 2-AG levels were also positively correlated after JZL184 treatment (Fig. 1L).

We next sought to determine the efficacy of pharmacological MAGL inhibition on anxiety-like behavior using the light-dark box assay, a well-validated ethologically relevant measure of anxiety (35, 36). Examination of time course data revealed JZL184 (3–15 mg kg⁻¹) increased light time, light distance, and total distance over time (Fig. 1M–O). JZL184 also dose-dependently increased total % light time, % light distance, but not total distance traveled (Fig. 1P–R). These data indicate JZL184 has a small but significant anxiolytic effect under basal conditions. For comparative purposes, we examined the effects of diazepam, and found as expected it increased light time and light distance in this assay (supplementary Fig. S2).

MAGL inhibition prevents stress-induced anxiety

Since stress is the major environmental risk factor for the development of mood and anxiety disorders (37), we sought to determine whether increasing 2-AG-mediated CB1 signaling could reduce stress-induced anxiety-like phenotypes. Mice tested in the light-dark box immediately after 30 min of restraint stress showed an anxiogenic response which was prevented by diazepam treatment, supporting the validity and utility of the model (supplementary Fig. S3). Importantly, 30 min of restraint stress significantly reduced AEA and increased 2-AG levels compared to control mice in the amygdala, but not PFC, and increased levels of corticosterone in the both brain regions (Fig. 2A–C). Moreover, we found a positive correlation between 2-AG levels and corticosterone in the amygdala (Fig. 2D).

We next asked whether pharmacological augmentation of 2-AG levels could compensate for stress-induced reductions in AEA signaling. Lipid analysis revealed that JZL184 (10 mg kg⁻¹) increased 2-AG levels (~28 nmols/g) compared to vehicle treated stressed mice (~19 nmols/g) without altering AEA levels in the brain (Supplementary Fig. S4). Behavioral results showed that systemic administration of JZL184 90 minutes prior to stress exposure

reduced stress-induced anxiety-like behavior compared to vehicle-treated mice (Fig. 2E–J). Analysis of time course data revealed stress exposure reduced light time, light distance, and total distance relative to non-stressed controls, and that JZL184 dose-dependently prevented these effects (Fig. 2E–G). Effects of stress to reduce total % light time, % light distance and total distance traveled were also prevented by JZL184 in a dose-dependent manner (Fig. 2H–J). As expected, inhibition of FAAH activity with PF-3845 (1 mg kg⁻¹) partially prevented the anxiogenic effects of restraint stress exposure (Fig. 2H–J purple bar). These data suggest that preventing stress-induced decreases in AEA levels prevents the emergence of stress-induced anxiety-like behaviors.

We next tested whether the anxiolytic-effects JZL184 after stress exposure were mediated via CB1 receptors. To test this, we co-treated mice with JZL184 (10 mg kg⁻¹) and CB1 receptor antagonist Rimonabant (2 mg kg⁻¹), or Rimonabant alone. Analysis of time course data revealed no significant effect of JZL184 on light time, light distance or total distance when co-treated with Rimonabant, compared to either Rimonabant alone, or stress alone (Supplementary Fig. S5). Taken together, these data support the hypothesis that pharmacological augmentation of 2-AG, and subsequent activation of CB1 receptors, can reduce stress-induced anxiety potentially by compensating for deficient AEA-mediated eCB signaling.

Direct CB1 receptor activation prevents stress-induced anxiety

To further validate the concept that stress-induced anxiety is partially mediated via reductions in AEA-mediated eCB signaling, and that mechanisms of engaging CB1 receptors other than 2-AG augmentation can compensate for this deficiency, we wanted to determine if direct CB1 receptor activation could prevent stress-induced anxiety in a manner similar to JZL184. To test this hypothesis, we treated mice with CB1 agonist CP55940 (0.001–0.3 mg kg⁻¹) 15 minutes before stress exposure and subsequent testing in the light-dark box. Behavioral analysis revealed CP55940 dose-dependently increased light time, light distance, and total distance (Fig. 3A–C). Analysis of total % light time and % light distance similarly revealed a dose-dependent anxiolytic effect of CP55940 (Fig. 3D–F). To examine whether CB1 agonism has any indirect effects on the eCB levels, we measured tissue content of AEA and 2-AG in the amygdala after CP55940 treatment (0.3 mg kg⁻¹). However, we did not find any significant effects of CP55940 treatment on the AEA and 2-AG levels in the amygdala (Supplementary Fig. S6). These data indicate that direct CB1 receptor activation can prevent the emergence of stress-induced anxiety in a manner similar to JZL184, and support the notion that alternate mechanisms of engaging CB1 receptors can compensate for stress-induced decreases in AEA-mediated eCB signaling.

Amygdala glutamatergic transmission predicts anxiolytic efficacy of 2-AG augmentation

Previous studies have suggested the anxiolytic effects of eCB augmentation may be due to CB1-dependent inhibition of glutamate transmission (38), and that stress-induced reductions in AEA levels contribute to excess glutamate release in the basolateral amygdala (BLA) (31, 39). Given the key role of the BLA in generating anxiety and fear responses (18), we hypothesized that pharmacological augmentation of 2-AG signaling could lead to reductions

in stress-induced excess glutamate release in the BLA, and that this could be related to its anxiolytic effects.

We performed whole-cell patch clamp recordings from visually identified BLA pyramidal neurons as previously described (26). In our first set of experiments we wanted to determine the potential effects of 2-AG augmentation of BLA neuron physiology. To do this, we incubated brain slices in 1 μ M JZL184 for 60 minutes prior to electrophysiological recordings (Fig. 4A). Under these conditions spontaneous excitatory postsynaptic current (sEPSC) frequency was significantly reduced (Fig. 4B–C); while no effects on sEPSC amplitude were observed (data not shown). JZL184 also increased intrinsic excitability and reduced action potential threshold without alerting the AP amplitude (Fig. 4D–G). Lastly, JZL184 increase membrane resistance, but did not affect membrane time constant, after hyperpolarization amplitude, or resting membrane potential (Fig. 4H–K).

We next determined if sEPSC frequency was affected by stress exposure and *in vivo* JZL184 treatment, and if these changes were related to behavioral measures of anxiety. Mice were treated with vehicle or JZL184 (10 mg kg⁻¹), exposed to restraint stress followed by behavioral testing in the light-dark box, immediately followed by electrophysiological analysis (Fig. 4L). Stress exposure increased sEPSC frequency relative to non-stressed mice (Fig. 4M–N) without affecting sEPSC amplitude (not shown). JZL184 treatment did reduce sEPSC frequency compared to vehicle treated stressed mice but did not reach statistical significance (Fig. 4O). However, in stressed mice treated with JZL184 a wide range of behavioral responses were observed, consistent with previous experiments (see Fig. 2), with some mice showing a clear anxiolytic effect defined as %light time >15%, while several mice did not (light time <15%). Separate analysis of these groups revealed that JZL184 treated mice that showed an anxiolytic response had significantly lower sEPSC frequency than stressed mice treated with vehicle or JZL184 treated mice that did not show an anxiolytic response (Fig. 4N–O). Moreover, linear regression revealed a significant correlation between % light time and sEPSC frequency across the population of mice analyzed (Fig. 4P). For these analyses each point represents the average frequency of all cells recorded from a single animal. This correlation remained significant when analyzing only vehicle treated non-stressed and stressed mice (Fig. 4Q), or JZL184 treated stressed mice (Fig. 4R). Taken together, these data indicate sEPSC frequency in the BLA is correlated with anxiety-like behavior measured in the light-dark box, and that the anxiolytic efficacy of JZL184 is correlated with reductions in BLA sEPSC frequency.

We also examined effects of PF-3845 treatment on stress-induced sEPSC frequency. We compared vehicle-treated stressed mice and mice showing clear anxiolytic effects (% light time >15%) after PF-3845 treatment. PF-3845 did not reduce sEPSC frequency compared to vehicle-treated stressed mice (Supplementary Fig. S7A). Linear regression revealed no correlation between % light time and sEPSC frequency in the vehicle and PF-3845 treated stressed mice (Supplementary Fig. S7B). However, PF-3845 treatment decreased sEPSC amplitude (Supplementary Fig. S7C), which suggests that PF-3845 and JZL-184 might exert anxiolytic actions via differential cellular mechanisms.

FAAH inhibition and THC prevents anxiety-like behavior caused by 2-AG depletion

Our data thus far suggests that pharmacological augmentation of 2-AG signaling and direct CB1 receptor stimulation can compensate for stress-induced deficiencies in AEA-mediated eCB signaling and prevent stress-induced anxiety. However, whether pharmacological AEA augmentation can reciprocally compensate for anxiety states induced by selective 2-AG depletion is not known. To explicitly test this hypothesis, we used irreversible DAGL inhibitor DO34 (50 mg kg⁻¹) to block endogenous synthesis of 2-AG (40), either alone or in combination with the PF-3845 (1 mg kg⁻¹). To validate our pharmacological treatments, we analyzed the effects of DO34 and DO34+PF-3845 on brain eCB levels. Administration of DO34 (50 mg kg⁻¹, i.p.) resulted in rapid and dramatic reductions in 2-AG and AA compared to vehicle-treated mice (Fig. 5A–B) (40); however, AEA levels were unaffected (Fig. 5C). Importantly, co-administration of DO34 and PF3845 resulted in selective increases in AEA and OEA above vehicle treatment, but maintained very low levels of 2-AG not different from DO34 treatment alone (Fig. 5A–D). These data confirm that FAAH inhibition only affected brain AEA, but not 2-AG levels, at the dose used. Analysis of time course data revealed DO34 decreased light time and light distance relative to vehicle treatment, and that co-administration of PF3845 (Fig. 5E–G) partially prevented these effects. Analysis of total % light time and % light distance similarly revealed a DO34 increased anxiety-like behaviors, while co-administration of PF-3845 (Fig. 5H–J) significantly prevented these effects.

We next determined if sEPSC frequency was affected by *in vivo* DO34 treatment. DO34 treatment increased sEPSC frequency relative to vehicle-treated mice (Supplementary Fig. S8A), which was correlated with % light time among mice treated with vehicle and DO34 (Supplementary Fig. S8B). However, DO34 did not affect sEPSC amplitude (Supplementary Fig. S8C). Moreover, PF-3845 treatment normalized DO34-induced increase in sEPSC frequency (Supplementary Fig. S8D–E) without affecting the sEPSC amplitude (Supplementary Fig. S8F). These data further support a tight relationship between anxiety-like state and BLA sEPSC frequency, and that sEPSC frequency can predict behavioral effects of bidirectional 2-AG modulation.

We and others have previously hypothesized that 2-AG deficiency associated with some forms of anxiety disorders could explain the high co-morbidity between cannabis use in some patients with anxiety disorders (26), and that a subset of patients with anxiety disorders that use cannabis do so to reduce their symptoms. To begin to test this hypothesis, we determined whether THC, the primary psychoactive component of cannabis, could reverse 2-AG-depletion-induced anxiety-like behaviors. Consistent with this hypothesis, THC (0.3 mg kg⁻¹) treatment reversed the anxiety-like behavior induced by DO34 treatment (Fig. 5K–P). These results support the conclusion that AEA augmentation and direct CB1 activation can reverse anxiogenic behavioral phenotype induced by acute 2-AG depletion.

Discussion

The main findings of this study are that 1) acute 2-AG augmentation reduces anxiety-like behaviors in the light-dark box assay, 2) acute restraint stress decreases amygdala AEA levels and increases anxiety-like behaviors, which can be prevented by 2-AG augmentation,

3) acute pharmacological 2-AG depletion increases anxiety-like behavior, which can be prevented by AEA augmentation and direct CB1 activation, and 4) amygdala glutamatergic transmission correlates with anxiety state and 2-AG-mediated reductions in amygdala glutamatergic transmission are correlated with anxiolytic efficacy. We conclude that, although AEA and 2-AG likely serve different physiological functions, a reciprocal redundancy exists between pharmacological AEA and 2-AG augmentation with regard to the modulation of anxiety-like behaviors. These data support development of eCB-based treatment approaches for mood and anxiety disorders and suggest a potentially wider therapeutic overlap between AEA and 2-AG augmentation strategies than previously appreciated.

We employed two approaches to investigate the functions of 2-AG signaling in the modulation of anxiety-like behaviors. First, facilitating 2-AG signaling through inhibition of 2-AG degrading enzyme, MAGL (34), and second, impairing 2-AG signaling by pharmacological blockade of the 2-AG synthesizing enzyme, DAGL (40). Consistent with previous reports, our results demonstrated that facilitation of 2-AG signaling not only reduced anxiety-like behaviors at basal conditions (28, 29, 41–43), but also reduced stress-induced anxiety-like behaviors (30, 44). These effects were also observed with the direct CB1 agonist CP55940. Moreover, the anxiolytic effects of JZL184 were mediated by CB1 receptors as CB1 antagonist blocked these effects. Previous studies have provided mixed results in this regard; while Busques-Garcia found the anxiolytic effects of MAGL inhibition were mediated by CB2 (28) but not CB1 receptors, Lim *et al*, Morena *et al*, Sciolino *et al*, and the present results clearly indicate a CB1-mediated mechanism (29, 30, 43).

Stress exposure can increase levels of 2-AG [(30, 33, 45–47) and current data], which are positively correlated with anxiety-like behaviors (43) and amygdala corticosterone levels (current data). Our data indicate that 2-AG augmentation reduces stress-induced anxiety-like behaviors suggest that physiological stress-induced increases in 2-AG represent a compensatory response aimed at counteracting stress-induced anxiety-like behaviors (albeit in an incomplete manner), and that pharmacological 2-AG augmentation bolsters this physiological response to more efficiently counteract the adverse effects of stress. We and others have also previously shown that stress and high levels of emotional arousal reduced AEA levels [(31, 39, 45, 48, 49), and current data], especially in the amygdala, and that AEA levels are inversely correlated with anxiety-like behaviors (16, 25, 50) as well as with stress induced corticosterone secretion (31). These data suggest a causal role for AEA deficiency in driving stress-induced anxiety. In this context, that pharmacological 2-AG augmentation reduces stress-induced anxiety via CB1 activation suggests a pharmacological redundancy between these two systems, such that augmentation of 2-AG can compensate for stress-induced deficiencies in AEA, thereby reducing adverse behavioral effects of acute stress exposure. We also tested the reciprocal hypothesis; specifically, that augmentation of AEA levels can counteract anxiety-states associated with 2-AG deficiency. Consistent with our prediction, 2-AG depletion with DO34 increased anxiety-like behavior, which was prevented by AEA augmentation via FAAH inhibition. Together, these data provide strong evidence of reciprocal redundancy between 2-AG and AEA signaling systems in the regulation of anxiety, whereby pharmacological augmentation of one eCB ligand can compensate for deficiencies in the other. Moreover, direct CB1 activation with THC also

prevented 2-AG deficiency-induced anxiety-like behavior. These data support the notion that some patients suffering from anxiety disorders associated with 2-AG deficiency may self-medicate with cannabis products to reduce their symptoms.

We have also investigated the neurophysiological mechanisms by which 2-AG could regulate stress-induced anxiety. We focused our studies on the BLA glutamatergic transmission based on previous studies suggesting the anxiolytic effects of eCB signaling are mediated via activation of CB1 receptors on forebrain glutamatergic neurons (38), that stress alters 2-AG signaling in the amygdala, and that amygdala specific modulation of eCB levels can modulate anxiety-like behaviors (43). We found that 2-AG augmentation can reduce BLA glutamatergic transmission, slightly increase BLA neuron excitability, and reduce AP threshold. While reductions in sEPSC frequency are consistent with the well-known role of 2-AG-CB1 signaling in the regulation of presynaptic neurotransmitter release (51), we are unsure of mechanism mediating the latter two postsynaptic effects, but the increase in excitability may be mediated by increases in membrane resistance. Acute stress increased sEPSC frequency onto BLA neurons, and sEPSC frequency was positively correlated with anxiety-like behavior. Combined with a large volume of data implicating increased amygdala activity in generating fear and anxiety-like responses (18, 20, 52, 53), increases in sEPSC are suggested to be causally related to the observed anxiety-like phenotype observed after stress exposure. In further support of this notion, mice treated with JZL184 that showed a robust anxiolytic response (Light time >15%) showed significantly reduced sEPSC frequency compared to stress alone, and not different from control mice. In contrast, mice that did not show a robust anxiolytic response to JZL184 treatment had sEPSC frequencies similar to that of vehicle-treated stressed mice. Indeed, among JZL184 treated mice, there was a significant positive correlation between sEPSC frequency and anxiety-like behavior such that mice with the lowest anxiety-like behavior had the lowest sEPSC frequency. These data suggest 2-AG inhibition of amygdala glutamatergic transmission may represent a synaptic mechanisms contributing to the anxiolytic effects of MAGL inhibition.

Our data are somewhat at odds with a recent report by Di et al., suggesting that in rats 2-AG signaling in the amygdala contributes to anxiety-like behavior in the open-field, but not elevated-plus maze, after restraint stress exposure (54). These authors did find, however, that blockade of CB1 receptors in the amygdala under control conditions increased anxiety-like behavior in the open-field, which is consistent with our data. Moreover, our studies are also consistent with other studies in rats indicating that 2-AG augmentation in the amygdala decreases anxiety-like behavior under non-stressed conditions (43), and that 2-AG levels in the amygdala correlate with HPA-axis habituation to repeated restraint stress (55). Therefore, overall it appears there is more support for the notion that amygdala 2-AG serves an anxiolytic function, however future studies should be aimed at conclusively addressing this somewhat controversial issue.

The limitations of the present study include the sole reliance on stress to cause an AEA deficiency state, which limits causal inferences regarding the role of reduced AEA in mediating increased anxiety-like behavior after stress. We attempted to utilize a genetic depletion model (NAPE-PLD KO mice), which have been suggested to reduce AEA levels (56). However, we did not observe reductions in brain AEA levels in these mice (data not

shown). Another limitation is that we only examined one brain region in our electrophysiological studies and all pharmacological studies were based on systemic delivery, which did not allow us to examine contributions of specific brain region in mediating the observed anxiolytic effects of eCB augmentation. In addition, although the choice of region and synapse was based on clear and compelling a priori evidence, it cannot be excluded that other synapses would show similar reductions in efficacy upon MAGL inhibition.

Despite these issues, our data provide novel insight into the dynamic interdependence between two eCB signaling systems in the modulation of anxiety-like behaviors and may have significant translational implications. For example, mounting data suggest that distinct psychiatric disorders are associated with selective alterations in eCB levels; i.e. patients with PTSD have reduced levels of circulating 2-AG (24), while some patients with depression may have reduced AEA levels (57). Therefore, if 2-AG augmentation is developed as a therapeutic approach for the treatment of affective disorders, our data suggest the efficacy would not be limited to pathological conditions under which 2-AG was deficient, but could also extend to conditions of AEA deficiency as well, and vice versa. The present data provide a solid foundation from which to test additional hypotheses regarding the pharmacological overlap between these systems in the regulation of emotional behaviors and affective psychopathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

These studies were supported in part by H. Lundbeck A/S, Copenhagen, Denmark (VUMC53005), and NIH Grants R01-MH107435 and F31-MH111103. PF-3845 was a gift from Pfizer. eCB measurements were conducted at the Vanderbilt Mass Spectrometry Research Center Facility and behavioral studies were carried out at the Vanderbilt Neurobehavioral Core Facility.

Financial Disclosures

Parts of this work were supported by H. Lundbeck A/S under a research contract with Vanderbilt University Medical Center (VUMC53005) to SP and LJM. NP is an employee of H. Lundbeck A/S.

References

1. Martin P. The epidemiology of anxiety disorders: a review. *Dialogues Clin Neurosci*. 2003; 5:281–298. [PubMed: 22034470]
2. Investing in treatment for depression and anxiety leads to fourfold return. WASHINGTON DC, USA: World Health Organization: Media Center; 2016 Apr 13. 2016
3. Reeves RR, Ladner ME. Antidepressant-induced suicidality: implications for clinical practice. *South Med J*. 2009; 102:713–718. [PubMed: 19488000]
4. Lader M, Kyriacou A. Withdrawing Benzodiazepines in Patients With Anxiety Disorders. *Curr Psychiatry Rep*. 2016; 18:8. [PubMed: 26733324]
5. Uhlenhuth EH, Balter MB, Ban TA, Yang K. International study of expert judgment on therapeutic use of benzodiazepines and other psychotherapeutic medications: IV. Therapeutic dose dependence and abuse liability of benzodiazepines in the long-term treatment of anxiety disorders. *J Clin Psychopharmacol*. 1999; 19:23S–29S.

6. Lader M. Benzodiazepine harm: how can it be reduced? *Br J Clin Pharmacol.* 2014; 77:295–301. [PubMed: 22882333]
7. Piomelli D. The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci.* 2003; 4:873–884. [PubMed: 14595399]
8. Kano M, Ohno-Shosaku T, Hashimoto-dani Y, Uchigashima M, Watanabe M. Endocannabinoid-mediated control of synaptic transmission. *Physiol Rev.* 2009; 89:309–380. [PubMed: 19126760]
9. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science.* 1992; 258:1946–1949. [PubMed: 1470919]
10. Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, et al. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun.* 1995; 215:89–97. [PubMed: 7575630]
11. Bedse G, Colangeli R, Lavecchia AM, Romano A, Altieri F, Cifani C, et al. Role of the basolateral amygdala in mediating the effects of the fatty acid amide hydrolase inhibitor URB597 on HPA axis response to stress. *Eur Neuropsychopharmacol.* 2014; 24:1511–1523. [PubMed: 25106694]
12. Patel S, Roelke CT, Rademacher DJ, Cullinan WE, Hillard CJ. Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. *Endocrinology.* 2004; 145:5431–5438. [PubMed: 15331569]
13. Morena M, Patel S, Bains JS, Hill MN. Neurobiological Interactions Between Stress and the Endocannabinoid System. *Neuropsychopharmacology.* 2016; 41:80–102. [PubMed: 26068727]
14. Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, et al. Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med.* 2003; 9:76–81. [PubMed: 12461523]
15. Bedse G, Romano A, Tempesta B, Lavecchia MA, Pace L, Bellomo A, et al. Inhibition of anandamide hydrolysis enhances noradrenergic and GABAergic transmission in the prefrontal cortex and basolateral amygdala of rats subjected to acute swim stress. *J Neurosci Res.* 2015; 93:777–787. [PubMed: 25581607]
16. Bluett RJ, Gamble-George JC, Hermanson DJ, Hartley ND, Marnett LJ, Patel S. Central anandamide deficiency predicts stress-induced anxiety: behavioral reversal through endocannabinoid augmentation. *Transl Psychiatry.* 2014; 4:e408. [PubMed: 25004388]
17. Gamble-George JC, Baldi R, Halladay L, Kocharian A, Hartley N, Silva CG, et al. Cyclooxygenase-2 inhibition reduces stress-induced affective pathology. *Elife.* 2016; 5
18. Gunduz-Cinar O, Hill MN, McEwen BS, Holmes A. Amygdala FAAH and anandamide: mediating protection and recovery from stress. *Trends Pharmacol Sci.* 2013; 34:637–644. [PubMed: 24325918]
19. Hermanson DJ, Hartley ND, Gamble-George J, Brown N, Shonesy BC, Kingsley PJ, et al. Substrate-selective COX-2 inhibition decreases anxiety via endocannabinoid activation. *Nat Neurosci.* 2013; 16:1291–1298. [PubMed: 23912944]
20. Hill MN, Kumar SA, Filipowski SB, Iverson M, Stuhr KL, Keith JM, et al. Disruption of fatty acid amide hydrolase activity prevents the effects of chronic stress on anxiety and amygdalar microstructure. *Mol Psychiatry.* 2013; 18:1125–1135. [PubMed: 22776900]
21. Marco EM, Rapino C, Caprioli A, Borsini F, Laviola G, Maccarrone M. Potential Therapeutic Value of a Novel FAAH Inhibitor for the Treatment of Anxiety. *PLoS One.* 2015; 10:e0137034. [PubMed: 26360704]
22. Moreira FA, Kaiser N, Monory K, Lutz B. Reduced anxiety-like behaviour induced by genetic and pharmacological inhibition of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH) is mediated by CB1 receptors. *Neuropharmacology.* 2008; 54:141–150. [PubMed: 17709120]
23. Seillier A, Giuffrida A. Inhibition of fatty acid amide hydrolase modulates anxiety-like behavior in PCP-treated rats. *Pharmacol Biochem Behav.* 2011; 98:583–586. [PubMed: 21421000]
24. Hill MN, Bierer LM, Makotkine I, Golier JA, Galea S, McEwen BS, et al. Reductions in circulating endocannabinoid levels in individuals with post-traumatic stress disorder following exposure to the World Trade Center attacks. *Psychoneuroendocrinology.* 2013; 38:2952–2961. [PubMed: 24035186]

25. Hill MN, Miller GE, Ho WS, Gorzalka BB, Hillard CJ. Serum endocannabinoid content is altered in females with depressive disorders: a preliminary report. *Pharmacopsychiatry*. 2008; 41:48–53. [PubMed: 18311684]
26. Shonesy BC, Bluett RJ, Ramikie TS, Baldi R, Hermanson DJ, Kingsley PJ, et al. Genetic disruption of 2-arachidonoylglycerol synthesis reveals a key role for endocannabinoid signaling in anxiety modulation. *Cell Rep*. 2014; 9:1644–1653. [PubMed: 25466252]
27. Jenniches I, Ternes S, Albayram O, Otte DM, Bach K, Bindila L, et al. Anxiety, Stress, and Fear Response in Mice With Reduced Endocannabinoid Levels. *Biol Psychiatry*. 2016; 79:858–868. [PubMed: 25981172]
28. Busquets-Garcia A, Puighermanal E, Pastor A, de la Torre R, Maldonado R, Ozaita A. Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses. *Biol Psychiatry*. 2011; 70:479–486. [PubMed: 21684528]
29. Sciolino NR, Zhou W, Hohmann AG. Enhancement of endocannabinoid signaling with JZL184, an inhibitor of the 2-arachidonoylglycerol hydrolyzing enzyme monoacylglycerol lipase, produces anxiolytic effects under conditions of high environmental aversiveness in rats. *Pharmacol Res*. 2011; 64:226–234. [PubMed: 21600985]
30. Lim J, Igarashi M, Jung KM, Butini S, Campiani G, Piomelli D. Endocannabinoid Modulation of Predator Stress-Induced Long-Term Anxiety in Rats. *Neuropsychopharmacology*. 2016; 41:1329–1339. [PubMed: 26361059]
31. Hill MN, McLaughlin RJ, Morrish AC, Viau V, Floresco SB, Hillard CJ, et al. Suppression of amygdalar endocannabinoid signaling by stress contributes to activation of the hypothalamic-pituitary-adrenal axis. *Neuropsychopharmacology*. 2009; 34:2733–2745. [PubMed: 19710634]
32. Patel S, Hillard CJ. Adaptations in endocannabinoid signaling in response to repeated homotypic stress: a novel mechanism for stress habituation. *Eur J Neurosci*. 2008; 27:2821–2829. [PubMed: 18588527]
33. Hill MN, McLaughlin RJ, Pan B, Fitzgerald ML, Roberts CJ, Lee TT, et al. Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. *J Neurosci*. 2011; 31:10506–10515. [PubMed: 21775596]
34. Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, et al. Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat Chem Biol*. 2009; 5:37–44. [PubMed: 19029917]
35. Kulesskaya N, Voikar V. Assessment of mouse anxiety-like behavior in the light-dark box and open-field arena: role of equipment and procedure. *Physiol Behav*. 2014; 133:30–38. [PubMed: 24832050]
36. Hascoet M, Bourin M, Nic Dhonnchadha BA. The mouse light-dark paradigm: a review. *Prog Neuropsychopharmacol Biol Psychiatry*. 2001; 25:141–166. [PubMed: 11263750]
37. McEwen BS. Mood disorders and allostatic load. *Biol Psychiatry*. 2003; 54:200–207. [PubMed: 12893096]
38. Rey AA, Purrio M, Viveros MP, Lutz B. Biphasic effects of cannabinoids in anxiety responses: CB1 and GABA(B) receptors in the balance of GABAergic and glutamatergic neurotransmission. *Neuropsychopharmacology*. 2012; 37:2624–2634. [PubMed: 22850737]
39. Gray JM, Vecchiarelli HA, Morena M, Lee TT, Hermanson DJ, Kim AB, et al. Corticotropin-releasing hormone drives anandamide hydrolysis in the amygdala to promote anxiety. *J Neurosci*. 2015; 35:3879–3892. [PubMed: 25740517]
40. Ogasawara D, Deng H, Viader A, Baggelaar MP, Breman A, den Dulk H, et al. Rapid and profound rewiring of brain lipid signaling networks by acute diacylglycerol lipase inhibition. *Proc Natl Acad Sci U S A*. 2016; 113:26–33. [PubMed: 26668358]
41. Aliczki M, Zelena D, Mikics E, Varga ZK, Pinter O, Bakos V, et al. Monoacylglycerol lipase inhibition-induced changes in plasma corticosterone levels, anxiety and locomotor activity in male CD1 mice. *Horm Behav*. 2013; 63:752–758. [PubMed: 23578952]
42. Kinsey SG, O'Neal ST, Long JZ, Cravatt BF, Lichtman AH. Inhibition of endocannabinoid catabolic enzymes elicits anxiolytic-like effects in the marble burying assay. *Pharmacol Biochem Behav*. 2011; 98:21–27. [PubMed: 21145341]

43. Morena M, Leidl KD, Vecchiarelli HA, Gray JM, Campolongo P, Hill MN. Emotional arousal state influences the ability of amygdalar endocannabinoid signaling to modulate anxiety. *Neuropharmacology*. 2016; 111:59–69. [PubMed: 27553121]
44. Sumislawski JJ, Ramikie TS, Patel S. Reversible gating of endocannabinoid plasticity in the amygdala by chronic stress: a potential role for monoacylglycerol lipase inhibition in the prevention of stress-induced behavioral adaptation. *Neuropsychopharmacology*. 2011; 36:2750–2761. [PubMed: 21849983]
45. Wang M, Hill MN, Zhang L, Gorzalka BB, Hillard CJ, Alger BE. Acute restraint stress enhances hippocampal endocannabinoid function via glucocorticoid receptor activation. *J Psychopharmacol*. 2012; 26:56–70. [PubMed: 21890595]
46. Evanson NK, Tasker JG, Hill MN, Hillard CJ, Herman JP. Fast feedback inhibition of the HPA axis by glucocorticoids is mediated by endocannabinoid signaling. *Endocrinology*. 2010; 151:4811–4819. [PubMed: 20702575]
47. Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley D, Mangieri R, et al. An endocannabinoid mechanism for stress-induced analgesia. *Nature*. 2005; 435:1108–1112. [PubMed: 15973410]
48. Patel S, Roelke CT, Rademacher DJ, Hillard CJ. Inhibition of restraint stress-induced neural and behavioural activation by endogenous cannabinoid signalling. *Eur J Neurosci*. 2005; 21:1057–1069. [PubMed: 15787710]
49. Rademacher DJ, Meier SE, Shi L, Ho WS, Jarrahian A, Hillard CJ. Effects of acute and repeated restraint stress on endocannabinoid content in the amygdala, ventral striatum, and medial prefrontal cortex in mice. *Neuropharmacology*. 2008; 54:108–116. [PubMed: 17675104]
50. Dlugos A, Childs E, Stuhr KL, Hillard CJ, de Wit H. Acute stress increases circulating anandamide and other N-acyl ethanolamines in healthy humans. *Neuropsychopharmacology*. 2012; 37:2416–2427. [PubMed: 22763622]
51. Castillo PE, Younts TJ, Chavez AE, Hashimoto-dani Y. Endocannabinoid signaling and synaptic function. *Neuron*. 2012; 76:70–81. [PubMed: 23040807]
52. Blair KS, Otero M, Teng C, Geraci M, Lewis E, Hollon N, et al. Learning from other people's fear: amygdala-based social reference learning in social anxiety disorder. *Psychol Med*. 2016; 46:2943–2953. [PubMed: 27476529]
53. Vytal KE, Overstreet C, Charney DR, Robinson OJ, Grillon C. Sustained anxiety increases amygdala-dorsomedial prefrontal coupling: a mechanism for maintaining an anxious state in healthy adults. *J Psychiatry Neurosci*. 2014; 39:321–329. [PubMed: 24886788]
54. Di S, Itoga CA, Fisher MO, Solomonow J, Roltsch EA, Gilpin NW, et al. Acute Stress Suppresses Synaptic Inhibition and Increases Anxiety via Endocannabinoid Release in the Basolateral Amygdala. *J Neurosci*. 2016; 36:8461–8470. [PubMed: 27511017]
55. Hill MN, McLaughlin RJ, Bingham B, Shrestha L, Lee TT, Gray JM, et al. Endogenous cannabinoid signaling is essential for stress adaptation. *Proc Natl Acad Sci U S A*. 2010; 107:9406–9411. [PubMed: 20439721]
56. Leishman E, Mackie K, Luquet S, Bradshaw HB. Lipidomics profile of a NAPE-PLD KO mouse provides evidence of a broader role of this enzyme in lipid metabolism in the brain. *Biochim Biophys Acta*. 2016; 1861:491–500. [PubMed: 26956082]
57. Hill MN, Miller GE, Carrier EJ, Gorzalka BB, Hillard CJ. Circulating endocannabinoids and N-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress. *Psychoneuroendocrinology*. 2009; 34:1257–1262. [PubMed: 19394765]

Figure 1. Acute MAGL inhibition elevates brain 2-AG signaling and reduces anxiety-like behavior

(A–F) Brain levels of 2-AG, AA, NA-Gly, 2-OG, AEA and OEA from mice treated with JZL184 at indicated dosages (3–15 mg kg⁻¹ intraperitoneally) after 3 h. (G–H) Brain and plasma levels of JZL184 after systemic exposure. (I–L) Plasma-brain correlations for JZL184, 2-AG and AEA. (M–R) Effects of JZL184 on (M) light time, (N) light distance, (O) total distance traveled by time course, and on (P) % light time, (Q) % light distance and (R) total distance travelled in 10 min. Significant F and P values from one-way and two-way ANOVA noted above bar graphs; **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 vs. vehicle by Holm-Sidak *post hoc* multiple comparisons test in bar graphs. Linear regression (solid line) with 95% confidence intervals (dashed lines) shown in figures. 2-AG, 2-arachidonoyl glycerol; 2-OG, 2-oleoylglycerol; AEA, N-arachidonylethanolamine; OEA, oleylethanolamine; AA, arachidonic acid and NAGly, N-arachidonoyl glycine. Data are presented as means ± s.e.m.

Figure 3. CB1 receptor agonist CP55940 reduces stress-induced anxiety-like behavior

(A–F) Effects of CP55940 (0.001–0.3 mg/kg) 45 min prior to behavioral testing on (A) light time, (B) light distance, (C) total distance traveled by time course, and on (D) % light time, (E) % light distance and (F) total distance travelled in 10 min. Data are presented as means $\pm$ s.e.m. Significant F and P values from one-way and two-way ANOVA noted above bar graphs; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. stress group by Holm-Sidak *post hoc* multiple comparisons test in bar graphs. Data are presented as means $\pm$ s.e.m.

Figure 4. Amygdala glutamatergic transmission predicts anxiolytic efficacy of 2-AG augmentation

(A) Schematic diagram depicts the recording of excitatory glutamatergic transmission in the basolateral amygdala (BLA) from brain slices incubated with JZL184 and vehicle. (B–C) JZL184 reduces spontaneous excitatory postsynaptic current (sEPSC) frequency (B). Representative traces of vehicle and JZL184 sEPSC (C). (D–G) JZL184 increased intrinsic excitability (D), reduced action potential threshold (E) and did not change action potential amplitude (F). Representative traces of vehicle and JZL184 action potentials at maximum current injection (G). (H–K) JZL184 increased membrane

input resistance (H) and did not affect membrane time constant (I), after hyperpolarization amplitude (J), or resting membrane potential (K). (L) Schematic diagram depicts the recording of excitatory glutamatergic transmission in the BLA of brain slices from mice systemically treated with JZL184 or vehicle. (M–O) Effects of stress exposure and JZL184 treatment on the sEPSC frequency of pyramidal neurons of BLA. Representative traces of vehicle and JZL184 sEPSC (N). (P–R) Correlation between % light time and sEPSC frequency of BLA pyramidal neurons of vehicle treated non-stressed, stressed and JZL184 treated stressed mice (P), vehicle treated non-stressed and stressed mice (Q) and JZL184 treated stressed mice [$<15\%$ LT and $>15\%$ LT; (R)]. Significant F and P values from *t* test and one way ANOVA noted above bar graphs; * $p<0.05$, ** $p<0.01$, *** $p<0.0001$ vs. control group by Holm-Sidak *post hoc* multiple comparisons test in bar graphs. Linear regression (solid line) with 95% confidence intervals (dashed lines) shown in figures. Data are presented as means $\pm$ s.e.m.

Figure 5. FAAH inhibition and THC administration attenuate 2-AG deficiency-induced anxiety-like behavior

(A–D) 2-AG, AA, AEA and OEA brain levels from the mice treated with DO34 and DO34+PF-3845. (E–J) Effects of DO34 and DO34+PF-3845 on (E) light time, (F) light distance, (G) total distance traveled by time course, and on (H) % light time, (I) % light distance and (J) total distance travelled in 10 min. (K–P) Effects of DO34 and DO34+THC on (K) light time, (L) light distance, (M) total distance traveled by time course, and on (N) % light time, (O) % light distance and (P) total distance travelled in 10 min. Significant F and P values from one-way and two-way ANOVA noted above bar graphs; *p<0.05, ***p<0.001 vs vehicle and #p<0.05, ##p<0.01, ###p<0.001 vs DO34 by Holm-Sidak *post hoc* multiple comparisons test in bar graphs. 2-AG, 2-arachidonoyl glycerol; AA, arachidonic acid; AEA, N-arachidonyl ethanolamine and OEA, oleoylethanolamine. Data are presented as means $\pm$ s.e.m.