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ORIGINAL ARTICLE

Central anandamide deficiency predicts stress-induced anxiety: behavioral reversal through endocannabinoid augmentation

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Stress is a major risk factor for the development of mood and anxiety disorders; elucidation of novel approaches to mitigate the deleterious effects of stress could have broad clinical applications. Pharmacological augmentation of central endogenous cannabinoid (eCB) signaling may be an effective therapeutic strategy to mitigate the adverse behavioral and physiological consequences of stress. Here we show that acute foot-shock stress induces a transient anxiety state measured 24 h later using the light–dark box assay and novelty-induced hypophagia test. Acute pharmacological inhibition of the anandamide-degrading enzyme, fatty acid amide hydrolase (FAAH), reverses the stress-induced anxiety state in a cannabinoid receptor-dependent manner. FAAH inhibition does not significantly affect anxiety-like behaviors in non-stressed mice. Moreover, whole brain anandamide levels are reduced 24 h after acute foot-shock stress and are negatively correlated with anxiety-like behavioral measures in the light–dark box test. These data indicate that central anandamide levels predict acute stress-induced anxiety, and that reversal of stress-induced anandamide deficiency is a key mechanism subserving the therapeutic effects of FAAH inhibition. These studies provide further support that eCB-augmentation is a viable pharmacological strategy for the treatment of stress-related neuropsychiatric disorders.

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INTRODUCTION

Stress is a major environmental risk factor for the development of mood and anxiety disorders.[1,2] Understanding the neurobiological mechanisms by which stress is translated into psychopathology is essential to developing novel therapeutic approaches for the treatment of affective disorders. Current clinically utilized pharmacological treatments for affective disorders are primarily based on augmenting monoaminergic transmission, but there is an increasing appreciation of the role of neuropeptides, cytokines and bioactive lipids in the pathophysiology of mood and anxiety disorders.[3] These non-monoamine-based modulators of mood and anxiety are promising targets for novel therapeutic approaches to treating affective disorders. In particular, multiple studies have demonstrated that pharmacological augmentation of central endogenous cannabinoid (eCB) signaling represents a promising approach to the treatment of mood and anxiety disorders.[4–7]

eCBs are lipid signaling molecules produced in the nervous system that exert biological actions primarily via the activation of cannabinoid receptors (CB_1 and CB_2). Anandamide (N-arachidonylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) are the two most well-studied eCB ligands. Converging preclinical studies strongly suggest that stress inhibits AEA signaling, and that pharmacological augmentation of eCB signaling may be a viable strategy to treat mood and anxiety disorders.[8–12] Systemic treatment with a direct CB_1 receptor agonist, however, produces both central and peripheral side effects.[13–16] Pharmacological inhibition of the AEA degrading enzyme, fatty acid amide hydrolase (FAAH), specifically augments AEA-mediated eCB signaling and has been shown to reduce unconditioned anxiety and despair behaviors in animal models in a CB_1 receptor-dependent manner.[7,17,18] More recently, genetic deletion and chronic pharmacological inhibition of FAAH have been shown to prevent emergence of some of the adverse behavioral effects of chronic stress.[19–21] Importantly, stress exposure decreases AEA levels in several limbic brain regions.[21–23] This suggests that deficits in AEA signaling contribute to stress-induced anxiety-like behavior, and that normalization of stress-induced AEA deficiency could be the mechanism subserving the anxiolytic effects of FAAH inhibition.

Here we sought to explicitly test the role of AEA signaling on acute stress-induced anxiety states. We first tested the effect of acute FAAH inhibition after the onset of stress-induced anxiety in two preclinical models of anxiety, the light–dark box test and the novelty-induced hypophagia (NIH) assay, which we have recently shown to be highly sensitive to eCB signaling.[4] Results from both models indicate that acute FAAH inhibition reverses the expression of anxiety-like behaviors induced by stress. We then analyzed the association between stress-induced AEA deficiency and anxiety state and found that stress-induced AEA deficiency significantly correlated with anxiety-like behavior. These data provide further support for the potential utility of FAAH inhibitors in the treatment of stress-related neuropsychiatric disorders.

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MATERIALS AND METHODS

Animals, foot-shock stress and drug treatments

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. Male ICR mice (4–7 weeks old) were housed on a 12:12 light–dark cycle with lights on at 0600 (Harlan, Indianapolis, IN, USA). All experiments were conducted during the light phase. Food and water were available *ad libitum*.

Foot-shock stress occurred 24 h before behavioral testing and consisted of six 0.7 mA foot-shocks delivered 1 min apart using a MED Associates fear-conditioning chamber (St. Albans, VT, USA). Each 2-s shock was preceded by a 30-s auditory tone. Drugs were administered 2 h before behavioral testing. The FAAH inhibitor PF-3845 (10 mg kg^{-1}, Cayman Chemical, Ann Arbor, MI, USA) and the CB_1 receptor antagonist rimonabant (3 mg kg^{-1}, NIMH Drug Supply Program) were dissolved in dimethylsulfoxide (Sigma Aldrich, Milwaukee, WI, USA) and intraperitoneally injected at a volume of 1 µl g^{-1}.

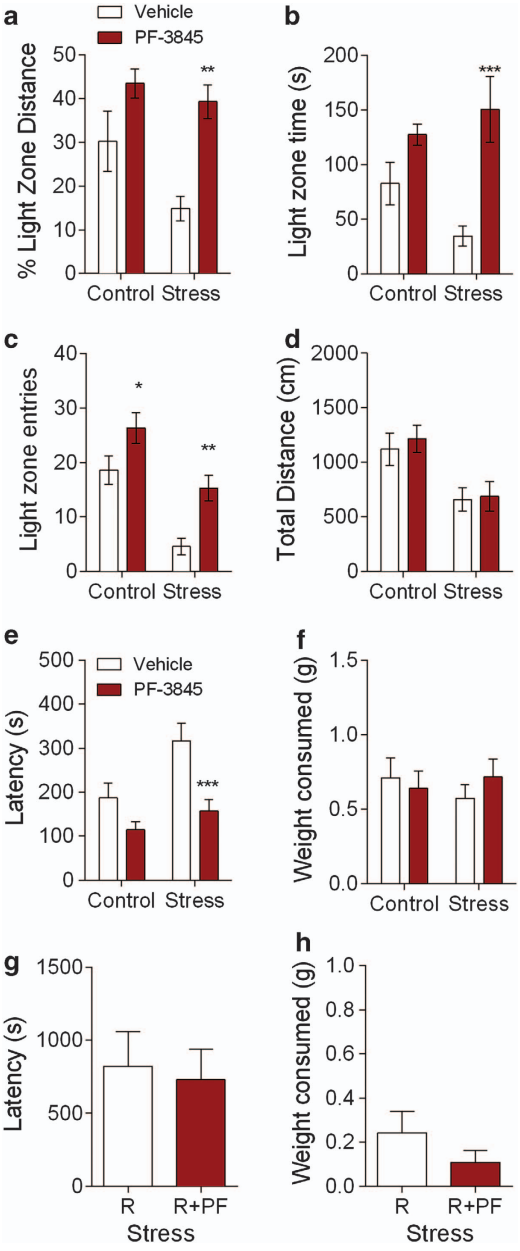

Figure 1. PF-3845 treatment reduces anxiety-like behaviors in the light–dark box and NIH assay. PF-3845 (maroon bars) reverses stress-induced anxiety in the light–dark box as compared with vehicle treatment (white bars) as measured by (**a**) distance traveled in the light zone as a percent of total distance traveled, (**b**) time in the light zone and (**c**) total number of light-zone entries. (**d**) PF-3845 also reverses the stress-induced increase in latency to first drink in the NIH assay (**e**) without affecting total food consumption (**f**). (**g**) Pharmacological blockade of CB_1 receptors via rimonabant treatment (R, white bars) prevents PF-3845 (maroon bars) from reversing stress effects on latency in the NIH assay. (**h**) PF-3845 does not significantly alter consumption in the NIH assay with rimonabant co-treatment. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significantly different from vehicle. NIH, novelty-induced hypophagia.

Behavioral assays

Light–dark box. Mice were individually placed into sound-attenuating chambers (27.9 × 27.9 cm; MED-OFA-510; MED Associates, St. Albans, VT, USA) containing dark box inserts that split the chamber into light (~25 lux) and dark (< 5 lux) halves (Med Associates ENV-511). Beam breaks from 16 infrared beams were recorded by Activity Monitor v5.10 (MED Associates) to monitor position and behavior during the 5-min testing period. Mice were stressed and drugs were administered as described above.

Novelty-induced hypophagia. The NIH test consisted of four training days in the home cage and one test day in a novel cage. Mice were singly housed for 4 days before training; cages were not changed for the duration of the experiment. Mice were habituated to testing rooms illuminated by red light (< 50 lux) for at least 30 min. After habituation, water was replaced with a highly palatable substance (liquid vanilla Ensure, Abbott Laboratories, Abbott Park, IL, USA) for 30 min during which latency to first consumption and total consumption were recorded. Foot-shock stress was administered immediately following the fourth training session and novel cage testing occurred 24 h later. Mice were habituated in red light for 60 min and then transferred to new, empty cages in a brightly lit room (~300 lux) with 30 min access to Ensure during which latency to drink and total consumption were recorded.

Mass spectrometry

One cohort of mice was killed by cervical dislocation and decapitation immediately following completion of the light–dark box test. Brains were then rapidly removed, frozen on a metal block in dry ice and stored at −80 °C until lipid extraction. Lipids from brain tissue were extracted via homogenization and sonication in 2 ml of acetonitrile containing 1000 pmol 2-AG-d_8 and 20 pmol AEA-d_8. The homogenate was centrifuged and the supernatant was removed and dried under nitrogen. Samples were then resuspended in 200 µl of methanol:water (50:50). Analytes were quantified using LC-MS/MS on a Quantum triple-quadrupole mass spectrometer in positive-ion mode using selected reaction monitoring. Detection of fatty acids was performed as previously described.[5,24]

Data analysis

Behavioral data were analyzed by *t*-test or analysis of variance (ANOVA) followed by *post hoc* Sidak's multiple comparison test unless otherwise specified. AEA levels and behavioral data were correlated by linear regression. All statistical analyses were conducted with Prism GraphPad 6 (San Diego, CA, USA). Results are shown as mean ± s.e.m. Statistical significance was set at $P < 0.05$.

RESULTS

To examine the effects of AEA augmentation on stress-induced anxiety we first determined the effects of the FAAH inhibitor PF-3845 on anxiety-like behavior in control mice and mice exposed to foot-shock stress 24 h before behavioral testing using the light–dark box. Stress induced anxiogenic effects in the light–dark box test. Two-way ANOVA revealed a significant effect of stress exposure on distance traveled in the light zone (Figure 1a, $F_{(1,35)} = 4.54$; $P = 0.04$) and number of light-zone entries (Figure 1c, $F_{(1,35)} = 28.27$; $P < 0.0001$), although its effect

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on light-zone time was not significant (Figure 1b). Conversely, ANOVA revealed PF-3845 treatment was anxiolytic in the light–dark box as shown by a significant increase in percent total distance traveled in the light zone (Figure 1a, $F_{(1,35)} = 16.9$; $P = 0.0002$), total light-zone time (Figure 1b, $F_{(1,35)} = 16.9$; $P = 0.0002$) and light-zone entries (Figure 1c, $F_{(1,35)} = 15.33$; $P = 0.0004$), but not total distance traveled (Figure 1d, $F_{(1,35)} = 0.23$; $P = 0.64$). *Post-hoc* analyses revealed that PF-3845 significantly increased percent light-zone distance ($P < 0.01$) and light-zone time ($P < 0.001$) in stressed mice. PF-3845 also significantly increased the number of light-zone entries in both stressed ($P < 0.01$) and control ($P < 0.05$) mice. No significant stress by PF-3845 interaction was observed for any anxiety measure in the light–dark box assay.

We then evaluated the effects of PF-3845 in control and stressed mice in a second preclinical model of anxiety, the NIH assay, which we have recently shown to be highly sensitive to tonic eCB signaling.[4] Two-way ANOVA revealed significant effects of stress (Figure 1e, $F_{(1,39)} = 7.6$; $P = 0.009$) and PF-3845 treatment (Figure 1e, $F_{(1,39)} = 13.77$; $P = 0.0006$) on feeding latency. Although there was no significant interaction between stress and PF-3845 treatment by ANOVA, *post hoc* analyses revealed that PF-3845 significantly reduced feeding latency in stressed ($P < 0.001$) but not control mice. Neither stress nor PF-3845 affected total food consumption in the NIH assay (Figure 1f).

To determine the mechanism responsible for the anxiolytic effect of PF-3845, we tested the effect of PF-3845 on stressed mice co-treated with rimonabant, a selective CB_1 receptor antagonist. Stressed mice were utilized due to the significant reduction in feeding latency produced by PF-3845 in stressed, but not control mice. Stressed mice co-treated with rimonabant and PF-3845 did not differ significantly in either latency or consumption from stressed mice treated with rimonabant alone (Figures 1g and h), confirming that PF-3845's anxiolytic effects in the NIH assay are mediated by CB_1 receptor activation. Although we did not directly test the effect of rimonabant relative to vehicle treatment here, we have previously demonstrated an anxiogenic effect of rimonabant in unstressed animals in the NIH assay.[4] Consistent with our previous work, comparison of feeding latencies between vehicle-treated stressed mice (~300 seconds; Figure 1g) and rimonabant-treated stressed mice (~800 seconds; Figure 1e) suggests that rimonabant maintains this anxiogenic effect after exposure to 1 day of foot-shock stress.

To investigate associations between stress-induced changes in eCB levels and anxiety-like behaviors, we analyzed whole brain eCB levels in control and stressed mice after completion of the light–dark box assay (~24 h after foot-shock stress) by mass spectrometry. Given that behavioral testing is itself a mild stressor, it is possible that exposure to the light–dark box test may have altered overall eCB content across all groups; however, this design allowed for direct analyses of the correlation between eCB levels and behavior in individual mice. Foot-shock stress reduced brain AEA levels in vehicle-treated (Figure 2a), $P < 0.05$ but not PF-3845 treated mice. In contrast, stress did not affect the levels of oleoylethananolamide or 2-AG (Figures 2b and c). As expected, PF-3845 treatment robustly increased AEA and oleoylethananolamide, but not 2-AG, relative to corresponding vehicle treatments in both control and stressed groups (Figures 2a–c, $P < 0.0001$ for all). We then performed a linear regression analysis of whole brain AEA levels and anxiety-like behaviors in the light–dark box of both control and stressed mice. This analysis revealed significant positive correlations between AEA levels and light-zone entries (Figure 3a), $r^2 = 0.0562$; $P = 0.0003$, percent light-zone distance (Figure 3c), $r^2 = 0.257$; $P = 0.031$, light-zone time (Figure 3e), $r^2 = 0.3$; $P = 0.018$, and total distance (Figure 3g), $r^2 = 0.3$; $P = 0.02$,

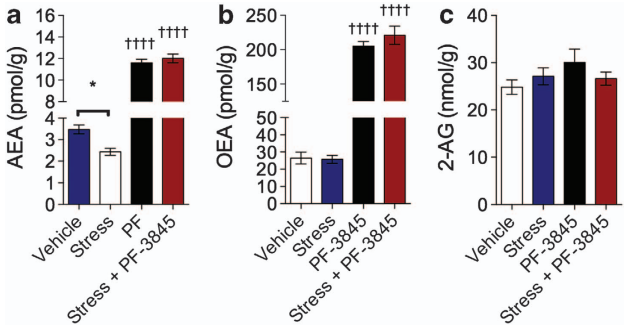

Figure 2. Stress reduces whole brain AEA levels while PF-3845 treatment increases AEA and OEA. Whole brain (**a**) AEA, (**b**) OEA and (**c**) 2-AG levels from mice immediately after completion of the light–dark box test (24 h after acute foot-shock stress and 2 h after drug treatment) demonstrate that stress decreases AEA and PF-3845 increases AEA and OEA, whereas neither stress nor PF-3845 treatment affects 2-AG. $^{*}P < 0.05$, $^{\dagger\dagger\dagger\dagger}P < 0.001$ significantly different from corresponding vehicle control. AEA, N-arachidonylethanolamine; 2-AG, 2-arachidonoylglycerol; OEA, oleoylethananolamide.

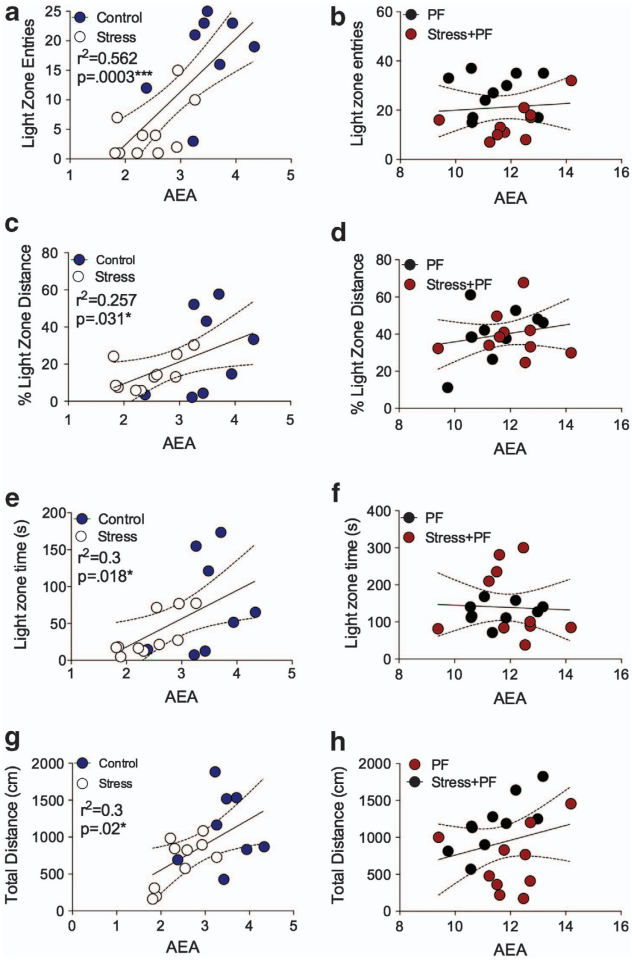

Figure 3. Whole brain decreases in AEA correlate with increased anxiety in the light–dark box test. Linear regression analyses reveal significant correlations between whole brain AEA levels in control and stressed mice and (**a**) light-zone entries, (**c**) light-zone distance traveled as a percent of total distance traveled, (**e**) light-zone time and (**g**) total distance traveled. After PF-3845 treatment, however, the same measures no longer correlate with AEA levels (**b**, **d**, **f** and **h**), suggesting a saturation of AEA effects on anxiety at supraphysiological levels. Linear regression (solid line) with 95% confidence intervals (dashed lines) shown in figures. AEA, N-arachidonylethanolamine.

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indicating that higher levels of AEA are associated with reduced anxiety-like behaviors. Linear regression of AEA levels and anxiety-like behaviors in control and stressed mice treated with PF-3845 did not reveal any significant correlations, suggesting a saturation of AEA effects on anxiety behaviors at supraphysiological levels (Figures 3b, d, f and h).

DISCUSSION

These data demonstrate that pharmacological elevation of AEA after stress, via acute FAAH inhibition with PF-3845, is able to reverse the anxiety-like behaviors typically exhibited after exposure to an intense, acute stressor in both the light–dark box and NIH assays. Interestingly, FAAH inhibition had little effect under control, non-stressed conditions, which is generally consistent with previous studies indicating that the anxiolytic efficacy of eCB-augmentation is enhanced by anxiogenic or aversive environmental contexts.[25–28] These studies are also consistent with previous data demonstrating that genetic deletion or pharmacological inhibition of FAAH during chronic stress exposure is able to prevent some of the adverse physiological and behavioral effects of stress.[19–21] Our studies extend these data to suggest that elevation of AEA could also be an effective treatment not only as a preventative measure, but also after stress-related psychopathology has begun to manifest. This ability to reverse already established psychopathologies is an essential component of potential novel therapeutics for clinical use.

Importantly, these studies demonstrate that whole brain levels of AEA are predictive of anxiety state, with decreased AEA corresponding to increased anxiety-like behavior in the light–dark test. Although it has been previously shown that stress decreases AEA,[19,29] this is the first direct evidence that lower levels of AEA in the central nervous system are correlated with greater expression of anxiety-like behaviors. This finding parallels recent studies showing that baseline anxiety inversely correlates with peripheral AEA content in human subjects[30,31] and that among individuals with posttraumatic stress disorder, those with lower peripheral AEA content exhibit more intrusive symptoms.[32] Further studies will be required to determine the validity of peripheral AEA levels as a biomarker for anxiety states. Importantly, our study also demonstrates that pharmacological augmentation of AEA signaling after stress exposure can reverse stress-induced anxiety, which is a necessary feature of novel therapeutics for stress-related psychopathology. Taken together, our findings strongly support the utility of AEA augmentation as a therapeutic approach for stress-related affective and anxiety disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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