Molecular Psychiatry
https://doi.org/10.1038/s41380-018-0215-1

ARTICLE

# Protective effects of elevated anandamide on stress and fear-related behaviors: translational evidence from humans and mice

Leah M. Mayo[1] · Anna Asratian[1] · Johan Lindé[1] · Lovisa Holm[1] · Daniel Nätt[1] · Gaëlle Augier[1] · Niclas Stensson[2] · Haley A. Vecchiarelli[3] · Georgia Balsevich[3] · Robert J. Aukema[3] · Bijar Ghafouri[2] · Primavera A. Spagnolo[4] · Francis S. Lee[5] · Matthew N. Hill[3] · Markus Heilig[1]

Received: 14 March 2018 / Revised: 25 June 2018 / Accepted: 28 June 2018
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## Abstract

Post-traumatic stress disorder (PTSD) is a common, debilitating condition with limited treatment options. Extinction of fear memories through prolonged exposure therapy, the primary evidence-based behavioral treatment for PTSD, has only partial efficacy. In mice, pharmacological inhibition of fatty acid amide hydrolase (FAAH) produces elevated levels of anandamide (AEA) and promotes fear extinction, suggesting that FAAH inhibitors may aid fear extinction-based treatments. A human *FAAH* 385C->A substitution encodes an FAAH enzyme with reduced catabolic efficacy. Individuals homozygous for the *FAAH* 385A allele may therefore offer a genetic model to evaluate the impact of elevations in AEA signaling in humans, helping to inform whether FAAH inhibitors have the potential to facilitate fear extinction therapy for PTSD. To overcome the challenge posed by low frequency of the AA genotype (appr. 5%), we prospectively genotyped 423 individuals to examine the balanced groups of CC, AC, and AA individuals ($n = 25$/group). Consistent with its loss-of-function nature, the A allele was dose dependently associated with elevated basal AEA levels, facilitated fear extinction, and enhanced the extinction recall. Moreover, the A-allele homozygotes were protected against stress-induced decreases in AEA and negative emotional consequences of stress. In a humanized mouse model, AA homozygous mice were similarly protected against stress-induced decreases in AEA, both in the periphery, and also in the amygdala and prefrontal cortex, brain structures critically involved in fear extinction and regulation of stress responses. Collectively, these data suggest that AEA signaling can temper aspects of the stress response and that FAAH inhibition may aid the treatment for stress-related psychiatric disorders, such as PTSD.

**Electronic supplementary material** The online version of this article (https://doi.org/10.1038/s41380-018-0215-1) contains supplementary material, which is available to authorized users.

✉ Markus Heilig
markus.heilig@liu.se

[1] Center for Social and Affective Neuroscience, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

[2] Pain and Rehabilitation Centre, Department of Medical and Health Sciences, Linköping University, Linköping, Sweden

[3] Hotchkiss Brain Institute and Mathison Centre for Mental Health Research and Education, Departments of Cell Biology and Anatomy & Psychiatry, University of Calgary, Calgary, Canada

[4] National Institute on Alcohol Abuse and Alcoholism and National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD, USA

[5] Institute for Developmental Psychobiology, Weill Cornell Medical College of Cornell University, New York, NY, USA

## Introduction

Post-traumatic stress disorder (PTSD) is a common, debilitating condition for which limited treatment options are currently available [1]. An impaired ability to extinguish fear memories when they no longer serve an adaptive purpose is a core feature of this condition [2]. Psychological PTSD treatments such as prolonged exposure therapy aim to promote fear extinction and have demonstrated clinical benefits, but the effects are frequently insufficient, and pharmacological tools to help extinguish fear memories are needed [3]. Medications that target the endocannabinoid (eCB) system may offer a mechanism to address this unmet need. Accumulating evidence suggests that the eCB anandamide (AEA) promotes fear extinction [4] and protects against the anxiogenic effects of stress through the activation of CB1 receptors [5–8]. Accordingly, in mice, extinction training leads to elevated AEA within the basolateral

amygdala [9], a brain region that is critical for emotional regulation and fear extinction learning [10], and that disruption of eCB signaling impairs fear extinction [9] and exacerbates neurobehavioral responses to stress [11].

Indiscriminate activation of CB1 receptors by exogenous agonists such as $\Delta^9$-tetrahydrocannabinol (THC) is associated with a potential for abuse liability and a range of negative consequences, including cognitive impairment [12]. A more selective strategy that avoids these challenges may be offered by the unique biology of the eCB system. AEA is synthesized on demand at the synapse, and its action is terminated through degradation by the serine hydrolase fatty acid amide hydrolase (FAAH) [13]. In experimental animals, potentiation of AEA via pharmacological FAAH inhibition selectively facilitates extinction learning [4, 14, 15]. In addition to targeting dysregulated fear responding, FAAH inhibition also mitigates anxiogenic effects of stress. This is likely related to the observation that stress causes a reduction in AEA in the amygdala [16–18], a key node of anxiety-promoting circuitry, and that this reduction can be prevented via FAAH inhibition [5]. Augmentation of AEA in experimental animals prevents the manifestation of anxiety-like behavior following stress, both acutely [6] and chronically [5, 19, 20]. FAAH inhibition does not appear to be anxiolytic per se, but protects against the anxiogenic effects of stress during times of heightened environmental aversiveness [5–8]. Collectively, these findings are consistent with a proposed role of the eCB system as a "stress buffer" [11].

FAAH inhibitors suitable for human use have been developed for non-psychiatric indications, such as inflammatory pain [21]. These programs have been discontinued due to the lack of efficacy in clinical trials, but have established selective that FAAH inhibitors as a class are safe, well tolerated, and lack abuse liability. This prompts the question whether FAAH inhibitors could be developed into PTSD therapeutics. Preliminary support for this notions comes from human studies that have examined the consequences of a loss-of-function *FAAH* 385C->A mutation (rs324420). This variant encodes an FAAH protein more susceptible to degradation and therefore is associated with an approximate 30% reduction of FAAH activity [22]. Studies that have examined the carriers of this variant generally support the notion that reduced FAAH activity may beneficially impact stress-related and fear-related behaviors in humans [4, 23–25]. However, these studies have largely relied on comparisons of *FAAH* 385C-homozygous and CA-heterozygous subjects. In *FAAH* 385CA-heterozygous individuals, potential protective effects of the A allele may be partially masked by expression of fully functional FAAH protein from the C allele. Although fewer than 5% of individuals of European descent are A homozygous, this group should be particularly informative in assessing the potential benefits of reduced FAAH activity at both the biochemical and behavioral level.

Here, we used a translational strategy in which human and mouse studies were used in concert to establish the biochemical and behavioral consequences of reduced FAAH activity. In the human study, we used a prospective genotyping strategy that allowed us to recruit balanced genotype groups, including a full group of AA homozygous individuals. In the mouse study, we used a humanized knock-in mouse model of *FAAH* C385A variation. This allowed us to examine the biochemical consequences of attenuated FAAH activity on AEA levels not only in the peripheral circulation, but also in the brain. In humans, we found that the *FAAH* 385A allele is associated with a gene-dose-dependent increase of basal AEA levels, facilitated fear extinction, and enhanced extinction recall. We also found that elevated AEA conferred via the A-allele protects against negative affective responding following stress exposure. We then used parallel human and mouse studies to demonstrate that AA homozygotes are protected against stress-induced decreases in circulating AEA, and in mice, also in stress-sensitive brain regions such as the amygdala and prefrontal cortex. Together, these findings provide compelling biochemical and behavioral support for the notion that FAAH inhibitors merit the evaluation as PTSD therapeutics.

## Methods and materials

### Methods and materials: human study

#### Participants

Participants ($n = 75$; 39/36 female/male) aged 18–35 years (mean = 24.4; SEM ± 0.4) were recruited from the Linköping University campus via flyers and online advertisements. A total of 423 individuals (CC = 251, AC = 147, AA = 25) were prospectively genotyped and screened, and included 25 of each genotype group (13/12 female/male). For inclusion and exclusion criteria, see Supplementary Information. Participants were paid 750 SEK (approximately 75 Euros) for their participation. All participants provided informed consent prior to participation, and the study protocol was approved by the Regional Ethical Review Board, Linköping.

#### Session overview

Participants completed two laboratory sessions (see Supplementary Fig. 1), each lasting for approximately 2 hours. All sessions started at noon or later and each subject came in at the same time of day on consecutive days. At the first session, participants completed a fear-conditioning task based on [26],

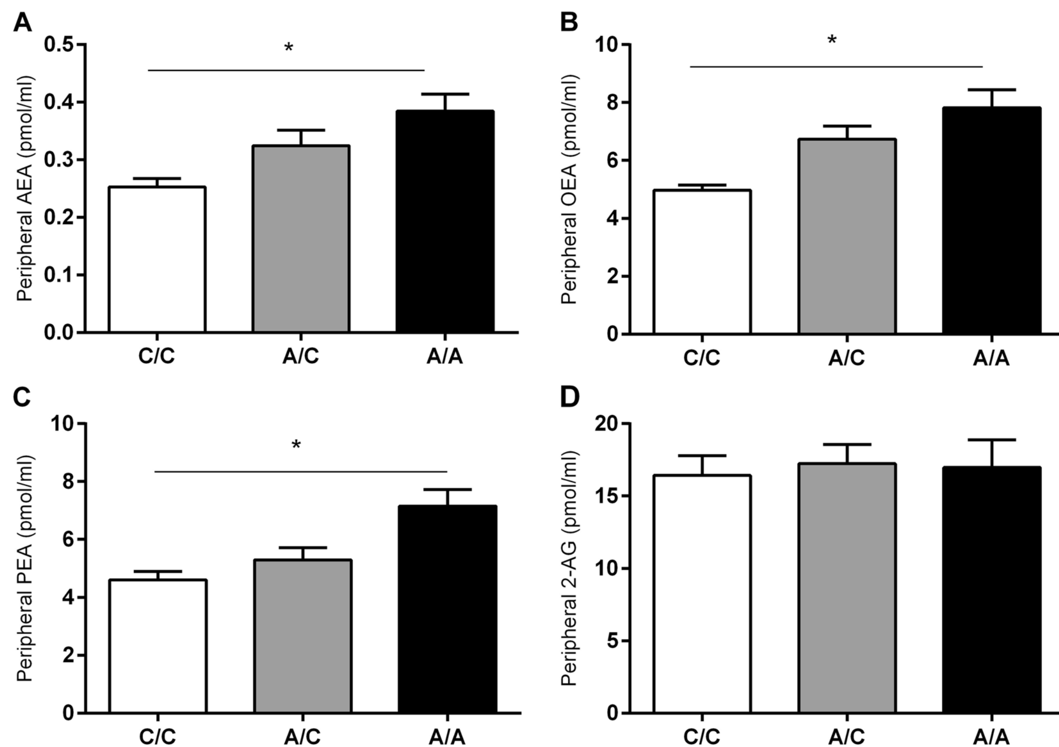


**Fig. 1** *FAAH C385A* influences the baseline peripheral levels of molecules hydrolyzed by FAAH in humans. **a** The A allele was associated with a gene-dose-dependent increase in basal peripheral AEA, as well as other molecules hydrolyzed by FAAH, including OEA (**b**) and PEA (**c**). **d** There was no effect of *FAAH C385A* on 2-AG, which is degraded in a manner distinct from FAAH. * $p < 0.001$; Bars represent means ± standard error of the mean (SEM) analyzed using one-way ANOVA with *Dunnet's* test for multiple comparisons using CC as the control group. Sample sizes: CC = 21, AC = 18, AA = 21

including habituation, acquisition, and extinction phases. Participants were then pseudorandomly assigned to the stress or control conditions, stratified by gender and genotype. Participants completed an affective image task, the control or stress procedure, and another affective image task. On day two, participants completed the remainder of the fear-conditioning procedure (recall of extinction and renewal of fear phases). They then completed the affective image task, the control or stress procedure (whichever one was *not* completed on day one), and another affective image task. Blood samples were collected before, immediately after, and following 20 min of recovery following stress and control procedures (see Fig. 1) to assess the levels of circulating eCBs and related compounds (AEA, 2-AG, OEA, and PEA) and cortisol. More detailed information regarding the sessions, task descriptions, and statistical analysis can be found in Supplementary Information.

## Psychophysiology

Upon arrival at both sessions, participants were outfitted with facial EMG sensors over the *zygomaticus major* ("zygomatic;" cheek), *corrugator supercilli* ("corrugator;" above the eyebrow), and *orbicularis oculi* ("orbicularis;" below the eye) muscles. Sensor application and data collection procedures were completed as previously reported [27]. Electrocardiography was assessed via disposable electrodes placed on the right supraclavicular fossa and mid-axillary on the left side of the abdomen, and eletrodermal activity was assessed via the thernar and hypothenar of the right hand. All data were collected using Biopac's MP150 Data Acquisition System (Biopac Systems, Inc, Camino Goleta, CA, USA).

## Fear-conditioning paradigm

The fear-conditioning task, based on [26] but adapted to a fear-potentiated startle paradigm, consisted of five phases conducted over 2 days. Day 1 consisted of habituation (HAB), acquisition (ACQ), and extinction (EXT) phases, with EXT divided into early (first four trials) and late (last four trials) phases. Day 2 consisted of recall of fear extinction (RCL) and renewal of fear responding (RNW). Throughout all phases, the eyeblink component of the startle response was measured following a blink-eliciting auditory stimulus ("startle probe"). The task included two "contexts;" digital photographs of two different rooms (a reception waiting room and an office). Each room contained a lamp that changed colors, and specific lamp colors constituted the conditioned stimuli (CS+, CS-). The US was an aversive sound [28], modeled after the sound of nails across a chalkboard, with a duration of 3 s.

### Affective image task

Affective images were selected from the International Affective Picture System [IAPS; [29]]. Pictures were presented for 6 s followed by self-reported ratings of valence and arousal. Facial EMG recordings of the zygomatic and corrugator were assessed as the mean EMG amplitude during the 6 s picture presentation compared with the immediately preceding 1 s baseline. The effect of stress on non-specific muscle activity (i.e., muscle activity in the absence of a stimulus) was assessed via averaging the 1 s pre-stimulus baseline throughout the task, comparing before ("pre") to after ("post") stress and control. The effect of stress on response to affective stimuli was assessed via average the cumulative EMG response to each stimulus type (positive, neutral, and negative).

### Stress task

The Maastricht Acute Stress Test (MAST), a modified version of the classic cold presser task, is a quick and non-invasive approach to elicit robust autonomic and glucocorticoid stress response [30]. The MAST is a 10 min task consisting of alternating "hand immersion" (HI) trials and "mental arithmetic" (MA) trials performed aloud with negative socio-evaluative feedback. Psychophysiological measures (heart rate and electrodermal activity) were collected during the task, and self-reported ratings were collected upon completion. Blood samples were collected via an intravenous catheter before, immediately after, and after 20 min of recovery.

### Genotyping

DNA was extracted via standard protocols using the InstaGene matrix (Bio-Rad Laboratories, USA), and genotyping at the *FAAH C385A* locus (rs324420) was done with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, USA) in an Applied Biosystems 7500-Fast Real time PCR (Applied Biosystems, Foster City, CA, USA USA) according to the manufacturer's instructions.

### Ancestry informative makers

Blood DNA was extracted using PureLink Genomic DNA Kit (ThermoFisher Scientific, Hägersten, Sweden) according to the manufacture's protocol. Genotyping was done on a TaqMan SNP genotyping assay set of 96 ancestry markers previously reported [31] using an ABI 7900 Sequence Detection System real Time PCR systems and TaqMan Low Density Arrays (Applied Biosystems, Foster City, CA, USA). Missing values were considered as heterozygotes (30 of 7315 SNP assays spread across 25 of the 77 subjects; 21 subjects had one, three subjects had two, and one subject had three missing SNPs). Assigning the missing values this way was chosen, since discarding all SNPs with any missing value ($n = 79$ full SNP series) gave very similar results. Categorical principal component analysis (CatPCA) was applied on the final genotype dataset, and factor scores for the two first principal components were extracted and tested for differences between the treatment groups.

### Cortisol analysis

Cortisol levels were obtained from serum samples analyzed using the DetectX Cortisol Enzyme Immunoassay kit (Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's instructions.

### Mass spectrometric detection of eCBs

The eCBs (AEA and 2-AG) and NAEs (OEA, SEA, PEA) were extracted and analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS), as previously published [32] (see also, Supplementary Information). Briefly, 300 µL of serum was thawed and vortexed, and 30 µL of a mixture containing the deuterated internal standard (AEA-d4, OEA-d4, and PEA-d4 (50 nM)) and 2AG-d5 (1000 nM) was added to each serum sample. C8 Octyl SPE columns (6 mL, 200 mg) (Biotage; Uppsala, Sweden) were used for lipid extraction. On the day of analysis, samples were reconstituted in 30 µL of LC mobile phase A. The injection volume was 10 µL. All standards and internal standards were purchased from Cayman Chemicals (Ann Arbor, MI, USA).

### Statistical analysis

Behavioral and biochemical analysis were carried out using one-way or repeated-measures analysis of variance (ANOVA), with genotype as a between-subjects factor, and Dunnet's post hoc tests to compare the reference group (CC) to the other genotypes (AC, AA), and an α level of 0.05. When possible, we reduced the dimensions (e.g., post–pre, stress–control). Sociodemographic and personality data were evaluated with a one-way ANOVA or chi-squared tests. A probability level of $<$ 0.05 was accepted as statistically significant.

## Methods and materials: mouse study

### Subjects

Male FAAH C385A mice, derived from the line generated in [23] and back-crossed on C57Bl6J strain for four generations, were bred in-house through heterozygote (AC)

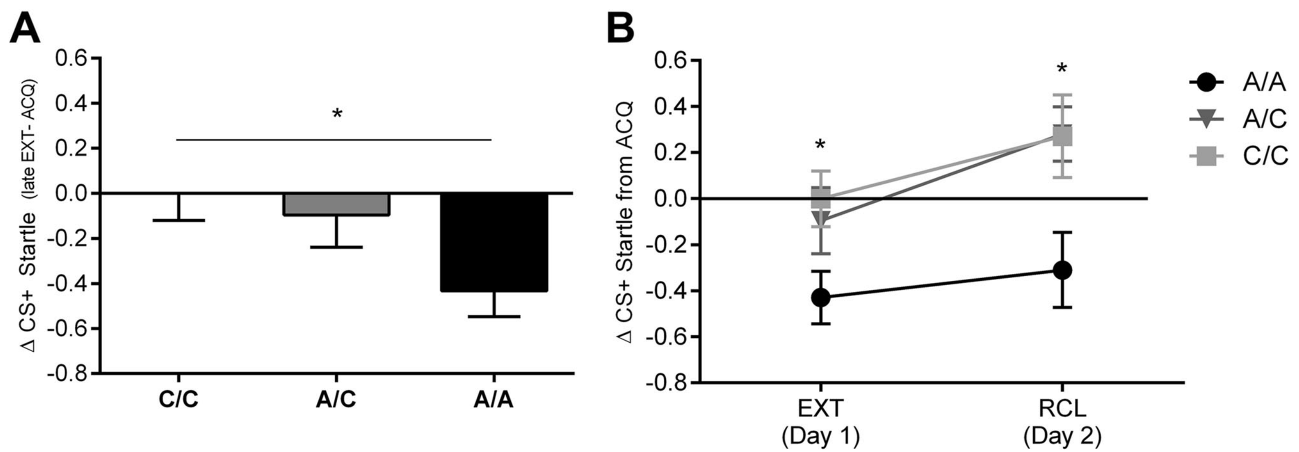


**Fig. 2** The *FAAH* A-allele is associated with facilitation fear extinction and enhanced extinction recall. **a** Carriers of the Aallele demonstrated greater extinction, shown as change in the standardized startle response to the fear-associated cue (e.g., CS+ startle/rest startle) from acquisition (ACQ) to late extinction (EXT; last four extinction trials). Here, more negative values denote greater extinction. **b** A-allele homozygotes also demonstrated enhanced recall of extinction learning (RCL; day 2) when tested 24 h after extinction learning (EXT; day 1). * $p < 0.05$ effect of genotype. Bars represent means ± SEM analyzed using one-way ANOVA with *Dunnet's* post hoc follow-up test. Sample sizes: CC = 24, AC = 25, AA = 24

male and heterozygote female (AC) breeding pairs. All experiments were approved by the University of Calgary Animal Care Committee and followed the guidelines from the Canadian Council on Animal Care.

### Stress exposure

Mice were exposed to 15 min of forced swim stress in room temperature (22+/- 1 ˚C) water, after which they were returned to their home cage. Control animals were removed at the same time as the stress animals, but were immediately sacrificed by rapid decapitation so to not be influenced by the stress of their cage mates [33]. Stressed mice were removed from the home cage 15 min following cessation of stress and rapidly decapitated. Prefrontal cortex and amygdala were dissected as previously described [18].

### Corticosterone analysis

Plasma was run in triplicate at a 1:500 dilution using a commercially available corticosterone ELISA (Caymen Chemical) as per the manufacturer's protocol.

### eCB quantification in animal studies

Lipid extractions were carried out as previously described [34, 35]. Briefly, frozen brain tissue was briefly weighed and then manually homogenized (with a glass rod) in borosilicate glass culture tubes containing 2 mL of acetonitrile with 5 nmol of d8-2-AG, 5 pmol of d8-AEA, 40 pmol d4-PEA, and 40 pmol d4-OEA. For plasma eCB levels, 200 µL of plasma was added directly to acetonitrile with the same preparation of internal standard as the tissue samples. All other steps of processing for plasma were identical to those for tissue (see Supplementary Information). Analysis in mass spectrometry was performed exactly as previously described [35].

## Results

The distribution of genotypes in the screened population (251 CC, 147 AC, and 25 AA) did not differ from the Hardy–Weinberg equilibrium (C allele frequency = 0.77; A allele frequency = 0.23; $X^2 = 0.32$; $p = 0.85$). The AIM panel identified two ancestry factors that did not differ between the genotype groups, and the groups did not differ on any demographic measure (Supplementary Table 1).

### *FAAH C385A* variation is associated with a gene-dose-dependent increase in basal AEA

The *FAAH* 385A variant is predicted to produce a FAAH protein more sensitive to proteolytic degradation, resulting in decreased FAAH activity and increased AEA levels [22]. However, the biochemical confirmation to date is limited [25, 36, 37], especially in regard to healthy humans. We found a robust, gene-dose-dependent effect of the A allele on baseline peripheral levels of AEA ($F(2,59) = 7.92$, $p = 0.001$; post hoc tests: AC vs. CC $p = 0.038$; AA vs. CC $p < 0.001$) (Fig. 1a) in humans. Additional analyses further supported that elevated AEA levels were caused by attenuated degradation by FAAH, since similar effects were seen for other molecules hydrolyzed by FAAH, including OEA ($F(2,5) = 7.79$, $p = 0.001$; post hoc tests: AC vs. CC $p = 0.070$; AA vs. CC $p < 0.001$) (Fig. 1b) and PEA

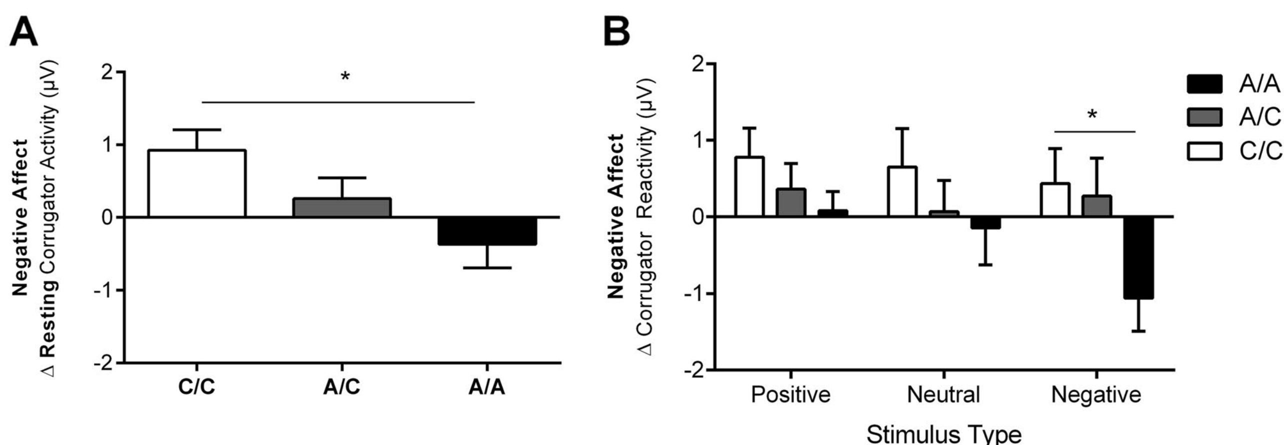


**Fig. 3** The A-allele protects against the negative consequences of stress. **a** A-allele was associated with a gene-dose-dependent reduction in stress-induced negative affect, as measured via the change in the resting corrugator activity due to stress compared with control. **b** Stress resulted in a net increase in negative affect, as indexed by greater corrugator reactivity, in response to all stimuli (e.g., positive, neutral, and negative images) in C-allele homozygotes. However, A-allele homozygotes demonstrated *reduced* negative affect specifically in response to negative stimuli following stress. * $p \leq 0.05$; Bars represent means ± SEM analyzed using one-way ANOVA with *Dunnet's* post-hoc follow-up test with CC as the control group. Sample sizes: CC = 24, AC = 25, AA = 24

($F(2,59) = 0.9.34$, $p < 0.001$; post hoc tests: AC vs. CC $p = 0.22$; AA vs. CC $p < 0.001$) (Fig. 1c). In contrast, there was no genotype effect on the levels of 2-AG ($F(1,58) = 0.24$, $p = 0.79$) (Fig. 1d), which is degraded in an FAAH-independent manner, further pointing to reduced FAAH activity as a causal factor behind the elevated AEA levels. The A allele produced similar effects on AEA levels in the peripheral blood of mice ($F(2, 14) = 3.78$; $p < 0.05$; post hoc tests: AC vs. CC $p < 0.02$; AA vs. CC $p < 0.05$; Fig. 4c; see also [23]); consistent with the human data, there was no effect of genotype on 2-AG levels (Supplementary Fig. 4B). Thus, as hypothesized, we found a gene-dose-dependent effect of the A allele on the basal AEA levels and on other molecules metabolized by FAAH, but not on 2-AG. This is a biochemical phenotype that replicates, albeit with lower efficacy, the expected activity profile of pharmacological FAAH inhibition. We next sought to determine the behavioral consequences of elevated AEA on fear- and stress-related behaviors.

## *FAAH 385A* selectively promotes extinction of conditioned fear

The eCB system is critically involved in the extinction of aversive memories [9], and elevation of AEA has been shown to facilitate fear extinction in mice [4, 23, 38]. Here, we determined the impact of the reduced-activity FAAH A-allele, which is associated with elevated AEA, on conditioned fear extinction in humans. Using a Pavlovian fear-potentiated startle paradigm, we found that the A-allele is associated with the facilitation of within-session fear extinction ($F(2,70) = 3.30$, $p = 0.041$; post hoc tests: AC vs. CC $p = 0.64$; AA vs. CC $p = 0.025$) (Fig. 2a), as well as enhanced recall of fear extinction when tested 24 h later ($F(2,69) = 4.55$, $p = 0.014$; post hoc tests: AC vs. CC $p = 0.69$; AA vs. CC $p = 0.01$) (Fig. 2b*; See also* Supplementary Fig. 2C). No differences were detected in the unconditioned startle response (Supplementary Fig. 2A), acquisition of fear conditioning (effect of CS: $F(1,72) = 38.86$, $p < 0.001$; cue * genotype, $p = 0.88$) (Supplementary Fig. 2B), response to the CS- during extinction ($p = 0.89$) (Supplementary Fig. 2D), or renewal of fear (effect of genotype: $p = 0.30$) (Supplementary Fig. 2E). Thus, the A allele does not influence innate fear expression or acquisition of conditioned fear, but is selectively associated with enhanced conditioned fear extinction and its recall. These findings are parallel and extend upon recent findings in humans [23], and represent a direct human translation of data previously obtained using humanized *FAAH C385A* knock-in mice [23] as well as pharmacological inhibition of FAAH [4].

## Elevated AEA conferred by *FAAH* 385A attenuates emotional, but not neuroendocrine, responses to stress

Elevated AEA conferred via reduced FAAH activity protects against the negative effects of stress [6–8, 17–20, 39, 40]. We therefore wanted to explore the consequences of elevated AEA conferred by the A-allele on emotional and neuroendocrine responses to stress in humans. We used an established stress task, the Maastricht Stress Task [30], and found that it robustly produced the expected increase in serum cortisol ($F(1,57 = 20.4$, $p < 0.001$; post hoc tests: AC vs. CC $p = 0.90$; AA vs. CC $p = 0.94$) (Fig. 4b), subjective stress ($F(1,71) = 381.8$, $p < 0.001$; AC vs. CC $p = 0.98$; AA

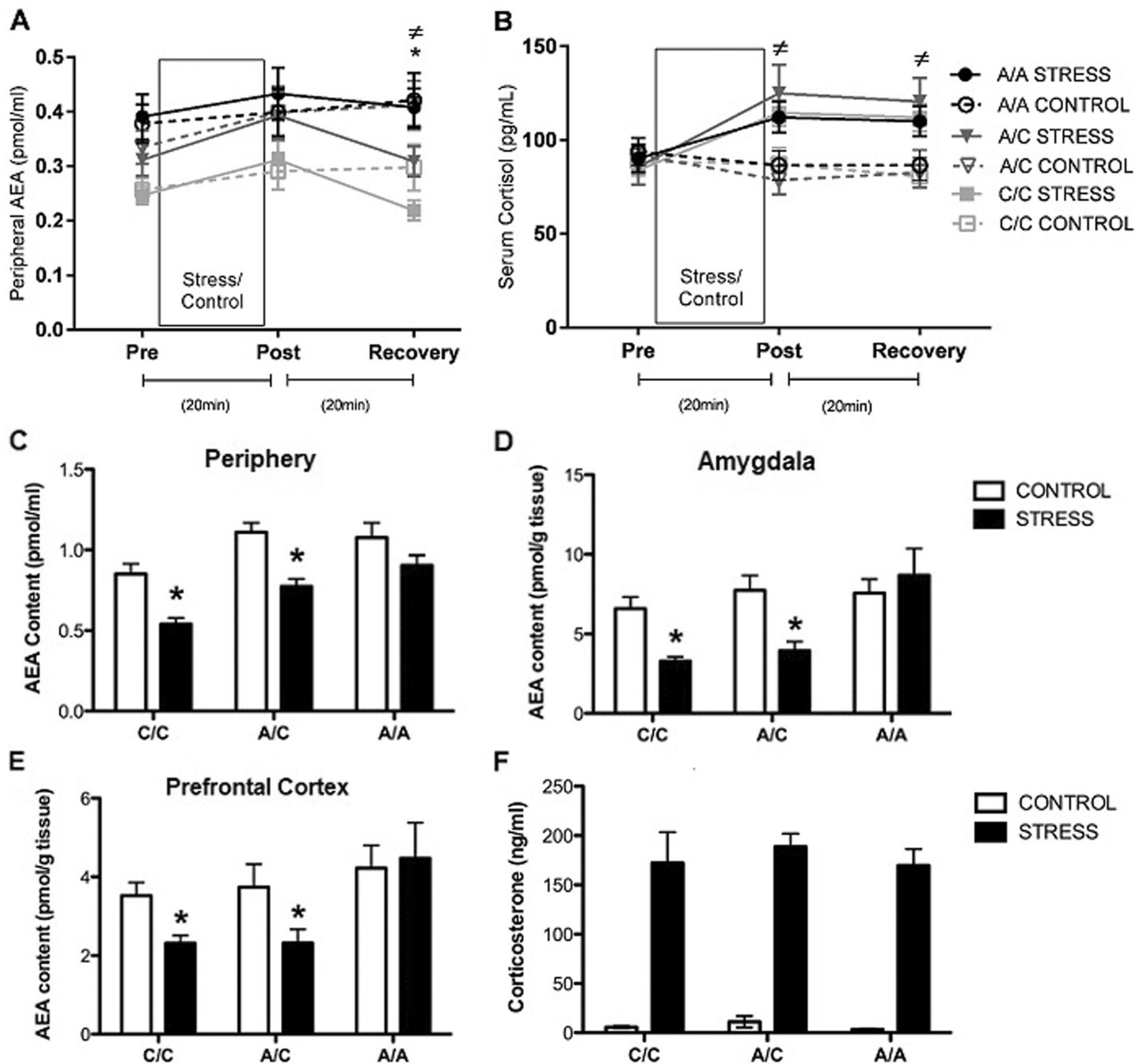


**Fig. 4** A-allele homozygotes are protected against stress-induced decreases in AEA across species. **a** As compared with the control session, stress elicited a significant reduction in peripheral AEA in humans during recovery, but this effect was absent in AA homozygotes. **b** While stress elected a robust increase in cortisol, there was no genotypic difference in the neuroendocrine response to stress at any time point. **c** Similar to humans, stress elicited a decrease in AEA in the peripheral blood of mice, but this effect was absent in AA mice. Stress also produced the decreased AEA in the **d** amygdala and **e** prefrontal cortex of CC and AC mice, but again, not in AA mice. **d** Stress elicited a significant increase in peripheral corticosterone, but similar to humans, there was no effect of genotype. **a**, **b** $\neq p < 0.05$ for stress vs. placebo session at specific time point; $*p < 0.05$ effect of genotype; Sample sizes: CC = 21(AEA)/20(Cort); AC = 18(AEA)/17 (Cort); CC = 21. **c–f** $* p < 0.05$ effect of stress. Sample size: $n = 6–13$ per group. Bars represent means ± SEM analyzed using one-way ANOVA with *Dunnet's* post-hoc follow-up test

vs. CC $p = 0.89$), and physiological arousal ($F(1,66) = 141.6$, $p < 0.001$, AC vs. CC $p = 0.24$; AA vs. CC $p = 0.09$) (Supplementary Fig. 3) in humans. However, there was no effect of genotype on any measure of stress (cortisol, $p = 0.95$; subjective stress, $p = 0.92$; physiological arousal, $p = 0.31$), nor was there an effect of genotype on basal cortisol ($p = 0.83$).

Animal studies suggests that FAAH inhibition is not anxiolytic in its own right, but instead mitigates the anxiogenic effects of stress [5–8]. Thus, we sought to determine whether the A allele influences affective responses in humans at baseline or following stress. To do so, we assessed affective responses to emotional images using facial electromyography (EMG) of the corrugator

("frown") and zygomatic ("smile") muscles, an objective and sensitive measure of affective response [29]. We obtained the facial EMG responses and self-reported ratings of valence and arousal in response to normatively rated affective images before and after stress and control procedures. No differences in baseline affect (e.g., responses to the first set of affective images) were detected via facial EMG (corrugator, $p = 0.61$; zygomatic, $p = 0.94$) or self-report ratings (valence, $p = 0.44$; arousal $p = 0.95$). However, stress produced an increase in the corrugator activity during rest (e.g., during the intertrial intervals, in the absence of any stimulus) that was inversely associated with the number of A alleles ($F(2,70] = 4.83$, $p = 0.03$; post-hoc tests: AC vs. CC $p = 0.21$; AA vs. CC $p = 0.03$; Fig. 3a). That is, CC individuals demonstrated the greatest increase in stress-induced corrugator activity at rest. This effect was not seen in the zygomatic ($p = 0.22$), ruling out a general effect on muscle tension. Thus, this increase in the corrugator activity is interpreted as a stress-induced increase in negative affect and is protected against by the A-allele.

Stress also influenced the responses *to* affective images. While CC individuals showed a net increasein negative affective responses to all stimuli after stress, this effect was absent in AA individuals ($F(2,140) = 3.75$, $p = 0.05$; post-hoc tests: AC vs. CC $p = 0.89$; AA vs. CC $p = 0.04$). Furthermore, AA individuals actually demonstrated *reduced* corrugator reactivity to negative affective images ($F(2,70) = 3.14$, $p = 0.05$; AC vs. CC $p = 0.98$; AA vs. CC $p = 0.04$) (Fig. 3b). Thus, A-allele homozygotes are not only protected against stress-induced increases in negative affect at rest, but also show reduced negative affect in response to negative emotional stimuli. As stress-induced declines in AEA signaling are believed to contribute to stress-induced changes in emotional responses [11] and AA homozygotes are resistant to stress-induced changes in negative affect, we next explored if AA individuals were protected against stress-induced declines in AEA signaling.

### *FAAH* 385A-allele homozygotes are protected against stress-induced decreases in AEA across species

We next examined the impact of stress on circulating levels of eCB molecules in humans and in mice humanized for the *FAAH* C385A polymorphism. Additionally, we determined if stress-induced changes in AEA signaling in the amygdala and prefrontal cortex of humanized *FAAH* C385A mice were modulated by a genotype in a parallel manner to any changes seen in the periphery. In humans, during recovery from the stressor task, the circulating levels of AEA were significantly reduced overall, but this reduction was absent in AA individuals ($F(1,59) = 7.01$, $p = 0.01$; main effect of genotype: $F(2,59) = 7.54$, $p = 0.001$; post-hoc tests: AC vs. CC $p = 0.03$; AA vs. CC $p = 0.001$; Fig. 4a). Thus, the A-allele homozygotes were protected against stress-induced decreases in peripheral AEA.

Mice carrying the A-allele showed remarkably similar biochemical consequences of stress. Mice of all genotypes were exposed to a 15 min forced swim stressor and then provided a brief recovery period (15 min) to parallel the approach taken in the human studies, after which AEA levels were measured in the peripheral circulation, the amygdala, and the PFC. Following exposure to stress, A-allele homozygotes specifically were protected against stress-induced decreases in circulating AEA ($F(2, 36) = 13.05$, $p < 0.01$; post hoc: CC stress vs. CC control $p < 0.01$; AC stress vs. AC control $p < 0.05$; AA stress vs. AA control $p > 0.05$) (Fig. 4c), as well as the amygdala ($F(2, 60) = 3.94$, $p < 0.03$; CC stress vs. CC control vs. $p < 0.05$; AC stress vs. AC controls $p < 0.05$; AA stress vs. AA controls: $p > 0.05$) (Fig. 4d) and prefrontal cortex (PFC) ($F(2, 50) = 3.47$, $p < 0.01$; CC stress vs. CC control vs. $p < 0.01$; AC stress vs. AC control $p < 0.05$; AA stress vs. AA control $p > 0.05$) (Fig. 4e). While stress increased 2-AG within the amygdala (Supplementary Fig. 6A) and PFC (Supplementary Fig. 6B), this did not differ between genotypes, nor were there detectable changes in 2-AG in the periphery (Supplementary Fig 5). Just as in humans, there was no effect of genotype on stress-induced changes in corticosterone (Fig. 4f).

Thus, in both species, A-allele homozygotes were protected against stress-induced decreases in peripheral AEA, and in mice, this protective effect extended to the key stress-sensitive brain regions, including the amygdala and prefrontal cortex. Furthermore, this effect was specific to AEA, as there was no effect of genotype on stress-elicited changes in 2-AG, which is degraded in an FAAH-independent manner, or glucocorticoid secretion.

## Discussion

Currently used pharmacotherapies for PTSD are geared more at mitigating the symptoms rather than mechanistically oriented to treat the underlying pathology, and their effect sizes are insufficient [1, 39, 41]. Consequently, there is a great need for novel PTSD medications that could target the core pathophysiology of this disorder, i.e., dysregulation of fear and stress responding. Animal studies have suggested that inhibition of the eCB-degrading enzyme FAAH may offer a novel mechanism to aid the current PTSD treatments. Here, we therefore used a human genetic model of decreased FAAH activity to provide initial proof-of-principle for this strategy. This was complemented by the use of a homologous humanized mouse model. Collectively, our data provide consistent translational support for

the feasibility of targeting FAAH with inhibitors as a treatment for PTSD.

Statistical associations between the candidate loci and complex traits, in particular when obtained in small case–control studies, suffer from multiple weaknesses, have frequently failed to replicate, and have largely been surpassed by more sophisticated approaches, such as genome-wide association studies (GWAS) [42]. In cases where a mutation has a direct, measurable biochemical consequence, however, a complementary strategy becomes possible. Prospectively determined allelic variation can, in these cases, be used as a proxy for directly—i.e., pharmacologically—manipulating that biochemical variable. This approach is what has allowed the emergence of precision medicine approaches, in which the predictive power can be sufficient to guide clinical decision-making at the single subject level, as exemplified by another metabolic enzyme, CYP2C19 [43].

To determine whether our human genetic model is appropriate to assess the therapeutic potential of FAAH inhibition, we therefore first established the link between *FAAH* genetics and eCB biochemistry. Our biochemical findings are consistent across humans and humanized mice carrying the same loss-of-function *FAAH* allele. In both species, we found that the *FAAH* 385A allele gene-dose-dependently increased the basal circulating AEA. Other eCBs degraded by FAAH were similarly affected, while 2-AG, which is degraded through a FAAH-independent pathway, was not. These findings establish a definitive biochemical consequence of variation at the *FAAH* C385A locus that can be detected peripherally. The use of humanized mice then allowed us to demonstrate that low FAAH activity also results in elevated AEA in brain areas of critical importance for fear-learning, anxiety, and stress responses. Because the humanized mouse model is implemented in inbred mice, i.e., on an invariant genetic background, these results also demonstrate that the consequences of *FAAH* C385A variation on AEA levels are independent of genetic variation elsewhere in the genome.

To our knowledge, this is the most extensive characterization of the biochemical consequences of *FAAH* gene variation on the eCB system. Our findings add to and expand on previous reports in clinical populations [25] and obese individuals [36, 37], as well as recent PET imaging findings, indicating that central FAAH levels are reduced in human *FAAH* 385A carriers [44]. The effect sizes observed across these studies may seem to contradict an emerging consensus from numerous GWAS, which suggests that individual effect sizes of common allelic variants should be very small [42]. We believe that this contradiction is only apparent. Generally, small effect sizes for common variants are well supported for complex disease phenotypes. This is not necessarily the case for simple biochemical endophenotypes that are proximal to the activity of the gene product. Once again, this is illustrated by established consequences of variation that affects the activity of CYP2C19 and other drug-metabolizing enzymes.

Having established that the *FAAH* 385A allele confers increased basal AEA levels, we then examined the behavioral consequences of this potentiation. We found that the A-allele is associated with facilitated fear extinction, replicating and extending previous studies with both mice and humans [23], and adding to the literature demonstrating similar effects produced by pharmacological inhibition of FAAH in rodents [4, 14, 15]. We used fear-potentiated startle to assess fear learning and extinction, while previous reports used skin conductance in humans and fear-induced freezing behavior in animals [23]. However, one previous study in rodents did use fear-potentiated startle as opposed to freezing behavior, and similar to our data, found that pharmacological elevation of eCB signaling enhanced the extinction of this behavioral measure as well [45]. The consistency of these findings demonstrates that this effect is robust and not limited to a single outcome measure or species. Importantly, this facilitation of extinction is not limited to within-session extinction, but is also evident 24 h later, corroborating that preclinical evidence that potentiated AEA may facilitate consolidation of emotional memories [46].

At a neural circuit level, extinction of fear is believed to involve functional coupling between the ventromedial prefrontal cortex (vmPFC) and the amygdala, whereby optimal extinction is achieved by increased activation of the vmPFC and deactivation of the amygdala. Disorders associated with impairments in fear extinction, such as PTSD, exhibit hypoactivity of the vmPFC and hyperactivity of the amygdala [47, 48]. Our findings of enhanced fear extinction in *FAAH* 385A-allele carriers could relate to alterations in neural activity within this circuit. Consistent with this hypothesis, activation of the eCB system by exogenously administered synthetic THC (oral dronabinol) prior to extinction training promotes recall of extinction when tested in a drug-free state 24 h later [49]. In addition to promoting fear extinction, THC administration was found to both reduce the amygdala reactivity to the previously fear-associated cue during extinction learning and increase the vmPFC activity during extinction recall testing [50]. Similar to the effects of THC, *FAAH* 385A-allele carriers have been found to exhibit blunted reactivity of the amygdala to threat cues [24]. As such, these data would suggest that elevations in AEA signaling at the CB1 receptor produced by the *FAAH* 385A allele promote fear extinction by increasing the activity in the vmPFC, reducing activity of the amygdala, or both. Animal studies support this differential response, as increased cannabinoid signaling has been found to reduce the firing rate of the amygdala neurons in vivo [51, 52],

while in the mPFC, eCB signaling predominately targets the inhibitory neurotransmission and could promote excitation of prefrontal neurons through a reduction in local GABAergic inhibition [53].

In addition to the activity of the vmPFC and amygdala per se, increased functional connectivity between these two structures acts to constrain the threat response [54, 55]. *FAAH* 385A-allele carriers exhibit increased structural and functional connectivity between the vmPFC and the amygdala [23, 56]. In contrast, the A-allele carriers did not differ from CC individuals in functional connectivity between the amygdala and dorsal anterior cingulate cortex, neurocircuitry associated with the expression of conditioned fear [57, 58]. This effect was replicated in humanized mice carrying the A allele, which demonstrated enhanced connectivity between the amygdala and the vmFPC, but no difference in connectivity between the amygdala and dorsomedial PFC [59]. Thus, elevated AEA conferred by reduced FAAH activity appears to promote the connectivity between the amygdala and vmPFC. This may facilitate the top-down control of fear responses necessary for fear extinction, providing a candidate mechanism for the potentiation of fear extinction observed in our study. Given that connectivity is impaired between the vmPFC and amygdala in PTSD and other anxiety-related disorders [60–62], the ability of AEA signaling to enhance the coupling of this circuit and promote fear extinction may provide a systems-level model of how targeting the FAAH activity could be a suitable pharmacotherapeutic option for PTSD.

The eCB system is increasingly recognized to play an important role in behavioral stress responses beyond extinction learning [11]. Preclinical studies have shown that stress produces a rapid induction of the FAAH activity that results in a reduction of the AEA signaling pool [16, 17, 63]. Accordingly, stress consistently reduces AEA within the amygdala [17, 18] and hippocampus [64, 65], while findings in the PFC vary depending on the type of stressor [63, 64, 66]. The reported effects of stress on AEA in humans have been less consistent [25, 67]. In the present study, we observed a stress-induced decrease in circulating AEA in both CC and AC individuals. In contrast, the A-allele homozygotes were protected against this consequence of stress exposure. We found the same effect in our humanized *FAAH* C385A mice; AA homozygotes were protected against the stress-induced decreases in circulating AEA observed in CC and AC genotypes. In the humanized mouse model, we also found that AA mice were protected against decreases in AEA in the stress-sensitive brain regions, including the amygdala and PFC. Thus, across species, we show that stress reduces peripheral AEA, and that the *FAAH* 385A allele protects against this effect. Furthermore, in mice, these effects extend to the brain. Collectively, our data suggest that *FAAH* 385AA results in the potentiation of AEA, and protection against stress-induced AEA decrease both in the periphery and brain. This is consistent with a recent PET study, which reported that *FAAH* variation influences binding of a FAAH-specific radioligand in the brain [44].

In animal models, pharmacological disruption or genetic deletion of FAAH prevents stress-induced decline in AEA content within the amygdala and the effects of stress on emotional behavior and reactivity [4, 5, 7, 19, 20, 39, 68]. Consistent with those preclinical reports, we found that after stress exposure, the A-allele carriers were protected against stress-induced enhancement of negative affect. Moreover, although we did not find genotype effects on self-reported ratings of affect or arousal, psychophysiological measures showed reduced negative affective responses to negative stimuli in AA homozygotes following stress exposure. Facial EMG, the method used to obtain these findings is a sensitive, objective measure of affect [29] that can be predictably modulated by pharmacological (e.g., [69]) and psychological (e.g., [70]) manipulations. Furthermore, in humans, the face broadcasts the affective states to others, profoundly influencing social interaction [71, 72]. The effects conferred by the A allele, an attenuation of stress-induced changes in response to negative affective stimuli, support the proposition that the eCB system can be viewed as a “stress buffer” [73].

We did not find any effect of elevated AEA levels associated with the *FAAH* 385A allele on stress-induced glucocorticoid secretion in humans or mice. Prior animal studies using FAAH inhibitors have yielded mixed results, with some reporting that inhibition of FAAH can dampen the HPA responses to stress [74, 75], while others not observing this effect [40, 76]. Thus, even with complete inhibition of the FAAH activity by an irreversible inhibitor, which results in dramatic elevations of AEA, effects on the HPA responses to stress are subtle at best and inconsistent. Given that the *FAAH* 385A homozygotes show only a partial loss-of-function for enzyme activity and modestly elevated AEA levels compared with what can be achieved by pharmacological inhibition of FAAH, it is not surprising that this was not sufficient to modify the HPA responses to stress in either the human or mouse AA homozygotes. Importantly, these data support the hypothesis that the impact of AEA signaling on stress, anxiety, and fear are likely due to direct modulation of neuronal excitability within cortico-limbic circuits and is not an indirect byproduct of alterations in hormonal reactivity to stress or aversive challenges itself. Future work using pharmacological tools will be required to elucidate if FAAH inhibition can dampen the stress-induced neuroendocrine changes in humans.

In summary, we present translational evidence that reduced FAAH activity results in elevated basal AEA and

protects against stress-induced decreases in AEA in the periphery and brain areas that process fear learning, anxiety, and behavioral stress responses. In humans, AA homozygotes show facilitated fear extinction and are protected against stress-induced increases in negative affect. Thus, even partial reduction of FAAH activity protects against negative biochemical and behavioral consequences of stress. Together, these observations provide a compelling rationale for evaluating FAAH inhibitors as an adjunctive pharmacotherapy for PTSD.

**Acknowledgements** The authors are grateful to Åsa Axén and Gisela Öhnström for their invaluable effort in participant screening, Matilda Areskoug for aid in data collection, and Dr. Margaret Wardle for continued technical assistance. The authors would like to acknowledge the Southern Alberta Mass Spectrometry Centre, located in and supported by the Cumming School of Medicine, University of Calgary, for their services in targeted liquid chromatography tandem mass spectrometry. This work was funded by the Swedish Research Council grant 2013-7434 to MH and grants from the Canadian Institutes of Health Research (CIHR) to MNH. HAV is a Vanier Scholar (CIHR) and receives studentships from the Alberta Innovates and BranchOut Neurological Foundation and GB is a Killam Scholar (CIHR).

## Compliance with ethical standards

**Conflict of interest** MNH receives salary support from CIHR in the form of a tier II Canada Research Chair. MNH has done consulting for both Pfizer and GW Pharmaceuticals.

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