Elevated Anandamide, Enhanced Recall of Fear Extinction, and Attenuated Stress Responses Following Inhibition of Fatty Acid Amide Hydrolase: A Randomized, Controlled Experimental Medicine Trial

Supplemental Information

Supplemental Methods and Materials

Visit	Screening	Visit 1	Visit 2	Follow-up phone call
Time point	Max. 14 days prior FDOT[a]	9 days (± 0) post FDOT[a]	10 days (± 0) post FDOT[a]	24 days (± 3 days) post FDOT[a]
Evaluation of inclusion/exclusion criteria	X			
Pregnancy test[b]	X			
Safety measures[c]	X	X	X	
Safety blood samples[d]	X		X	
Informed consent	X			
Blood sample, genotyping	X			
Urine sample, illicit drug screen[f]	X			
Randomization	X			
Distribution of study drug/placebo	X			
Self-report questionnaires (NEO-FFI, STAI-T)	X			
Behavioral tasks		X	X	
Psychophysiological measurements[e]		X	X	
Self-report questionnaires (STAT-S, PANAS, POMS)		X	X	
Blood samples (endocannabinoids, cortisol)		X	X	
Adverse event registration		X	X	X
Compliance control		X	X	
Collection of study drug/placebo			X	

Supplemental Table S1: Data collection procedures. [a]FDOT: First day of treatment, [b]Only for women of child-bearing potential, [c]blood pressure, heart rate, well-being and psychiatric symptoms, [d]AST, ALT, GGT, WBC with diff., Hb, MCV, TPK, LPK and CRP, [e], heart rate, respiration, skin conductance, facial EMG; [e]THC, amphetamine, methamphetamine, opioids, benzodiazepines, cocaine, buprenorphine, methadone, tramadol, oxycontin, fentanyl, and clonazepam.

Inclusion and Exclusion Criteria

Participants were required to be 18 years or older and provide informed consent. Exclusion criteria include: lifetime diagnosis of psychosis or bipolar disease, current axis 1 diagnosis; as determined by

a history, clinical examination and MINI interview carried out by appropriately trained staff; ongoing (within the last month) psychiatric medication; current (within the last month) use of illicit drugs, as identified using the Drug Use Disorder Identification Test (DUDIT) or a positive urine screen; co-medication with CYP3A inhibitors, CYP3A inducers or P-glycoprotein substrates; any other current medication or medical condition that in the judgment of the investigator could interfere with treatment; pregnancy or nursing. To be eligible, women of childbearing potential (WOCBP) must have a negative serum or urine pregnancy test prior to the start of study drug. WOCBP and males with WOCBP partners must agree to use a method of contraception that is highly effective for the duration of the study and for at least 28 days after the intake of the study drug.

Drug (PF-04457845)

PF-04457845 (Pfizer, Groton, VT, USA) is an orally available, highly selective covalent inhibitor of FAAH (1, 2). The pharmacokinetics have been characterized in healthy adult volunteers in single doses (dose range 0.1 to 40 mg) or in multiple doses (dose range 0.5 to 8 mg). Maximal plasma concentrations of the drug were reached within two hours of administration and the half-life is estimated to be 12 to 23 hours. Multiple dosing produces steady-state plasma concentrations by day 7 of dosing. The 4 mg daily dose was selected because it maximally inhibits FAAH and is well tolerated, while higher doses do not produce any detectable advantage. No serious adverse events were reported in any of the completed phase I studies, or clinical studies including patients with osteoarthritis or cannabis use disorder (1–5). Positron emission tomography has shown that a 1 mg dose of PF-04457845 effectively inhibits FAAH (>95%) in the human brain (6). Following multiple dosing of 4mg, the washout period is estimated to take 10 days.

The drug supply used in this study was originally designated for a clinical trial assessing the efficacy of PF-04457845 on co-morbid PTSD and alcohol use disorder (AUD) in women at the Karolinska Institute, Stockholm, Sweden (EudraCT 2014-002456-9). Drug and matched placebo were provided in bulk by Pfizer, to be packaged, labeled and released by a local contractor and the hospital pharmacy. Like all FAAH inhibitor trials, this trial was placed on a clinical hold after a report that the FAAH inhibitor BIA 10-2474 had caused serious toxicity including 1 death in a Phase 1 study (7, 8). These effects were later attributed to off-target toxicity of this specific compound (9), and the clinical hold was lifted. Meanwhile, however, the original study had been dropped, and replaced by a behavioral treatment trial that is currently ongoing (10).

When the clinical hold was lifted, and it was clear that PF-04457845 was safe to use in humans, the remaining drug was therefore re-purposed for the current study with approvals of the Swedish Medical Products Agency and concurrence from Pfizer. The drug was sent from the pharmacy at the Karolinska Hospital in Stockholm to be re-labeled by a contractor (Oriola; Stockholm, Sweden). The re-labeled medication was then sent to the pharmacy at University Hospital in Linköping, Sweden, and distributed to participants as previously detailed. Unfortunately, this involved 1 pharmaceutical company, two contractors, and two different hospital pharmacies.

In the current study, we initially monitored compliance by pill-count, and debriefed all subjects on their compliance during follow-up, which was part of the study to monitor safety. We then analyzed AEA levels as an objective biomarker of target engagement. In these data, approximately half (N = 15) of the participants randomized to the FAAHi group had unaffected AEA levels, while the remaining participants had levels that were on average 10-fold elevated. We then analyzed drug levels, and found that individuals with unaffected AEA levels also did not have detectable drug levels. Of note, we analyzed samples from all subjects (placebo and FAAHi-treated), and could confirm that no participants in the group randomized to placebo had received active drug.

Finally, we carried out an extensive audit, in which each participant was re-contacted and debriefed personally by the sponsor / PI. Participants were informed of the situation and asked whether they had any new information to provide to help account for the findings, with no penalty to themselves. While in some cases admitting to behaviors that could be considered problematic in the study (one case: THC use; two other cases: one or more episodes of binge drinking), they all ensured that their initial report on drug compliance was correct.

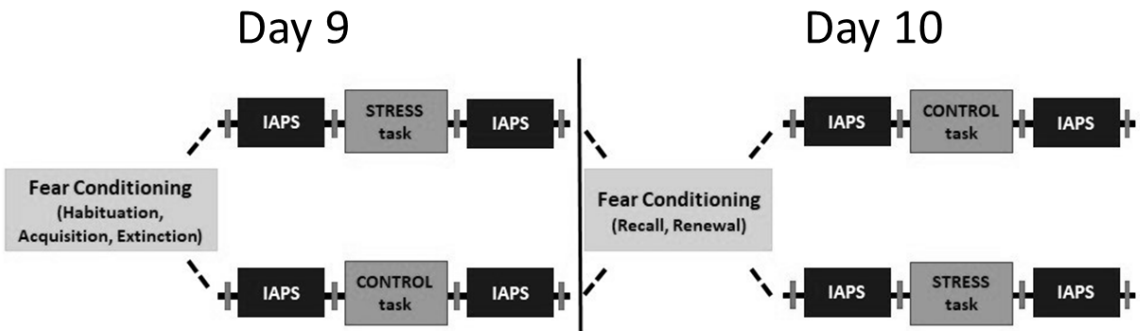
In conclusion, all subjects randomized to receive placebo were objectively confirmed to have received their allocated treatment, while only 16 subjects randomized to receive active medication received it, with the remainder of this group inadvertently receiving placebo due a packaging / labeling error.

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. Facial EMG sensors consisted of 4mm silver/silver chloride electrodes filled with electrode gel; two placed on each muscle location to form bipolar recording pairs. Recording electrodes were placed on muscles on the left side of the face and an 8mm ground electrode was placed on the forehead near the hairline. Sites were cleaned with alcohol and lightly abraded and any site with impedance over 20kΩ (measured with a Model 1089 MK III Checktrode; UFI, Morro Bay, CA, USA) was reapplied. EMG signals were amplified, filtered through a 10-500 Hz band pass and 50 Hz comb band stop filter, digitized at 1 kHz, re-filtered, rectified, and integrated over 20ms using EMG100C amplifiers, MP150 Data Acquisition system and Acqknowledge software from Biopac Systems (Biopac Systems, Inc, Camino Goleta, CA, USA). In all tasks, trials with excessive baseline activity or artefactual activations were identified and excluded by trained, blinded

raters. The number of trials excluded based on these factors ranged from 2.5 to 9.5% across muscle location (zygomatic, corrugator, orbicularis) and task.

Electrocardiography (ECG) was assessed via disposable Ag/AgCl snap gel electrodes placed at the right supraclavicular fossa and mid-axillary on the left side of the abdomen. Sites were cleaned with alcohol prior to electrode placement. Data were relayed to the Biopac ECG100C amplifier, filtered, and digitized at 1 kHz. Electrodermal activity (EDA) was assessed via two disposable Ag/AgCl contact snap electrodes (EL507; Biopac) pre-gelled with isotonic (0.5% chloride salt) gel placed on the thenar and hypothenar of the right hand. Data were relayed to a Biopac EDA100C amplifier, employing a constant voltage technique and sampling the absolute, direct current skin conductance at the rate of 20 samples per second. Non-specific skin conductance responses (SCRs) were obtained with an additional high pass filter applied to the EDA channel to remove the slow changes in skin conductance. SCR threshold was set at 0.2μs and responses with an amplitude of less than 10% of the mean were disregarded. SCR frequency, mean amplitude, and maximum amplitude during the 10min task period were collected for the control and stress tasks, compared to a 5min baseline period.



Supplemental Figure S1: Study schematic. Participants completed laboratory sessions on days 9 and 10 of dosing. All participants first completed fear conditioning tasks, including habituation, acquisition, and extinction. Then they completed an affective image task (IAPS), stress or control, and another affective image task. On day two, they underwent recall of fear extinction and renewal of fear. They again completed an affective image task (IAPS), stress or control (whichever was not completed on day 9), and another affective image task. Blood samples were taken via an indwelling intravenous catheter before and after IAPS and stress/control tasks. Participants were randomized to receive stress or control first.

Behavioral Tasks

Fear Conditioning Paradigm. The fear conditioning task is based on (11) but adapted to a fear-potentiated startle paradigm consisting of five phases conducted over two days as described in detail in (12). Briefly, day one consisted of habituation, acquisition (ACQ), and extinction (EXT) phases, with EXT divided into early (first four trials) and late (last four trials) phases. Day two consisted of recall of fear extinction (RCL) and renewal of fear responding (RNW). Throughout all phases, the eye-blink component of the startle response was measured following a blink-eliciting auditory stimulus ("startle probe"), e.g. 50 msec burst of white noise. Auditory stimuli were presented via Sennheiser Model HD-25 headphones.

The task included two 'contexts'; digital photographs of two different rooms (a reception waiting room and an office) which contained a lamp that changed color, with specific lamp colors constituting the conditioned stimuli (CS+, CS-). The US was an aversive sound modeled after the sound of nails across a chalkboard, with a duration of 3 sec (13). The ACQ phase took place in one of the two contexts (CTX+), with each CS presented eight times and the CS+ reinforced 100%. EXT took place 10 min after ACQ, in CTX- (the context not used in ACQ). RCL took place on day two in CTX- (e.g. the same context as EXT) followed by RNW in CTX+ (e.g. the same context as ACQ). Both RCL and RNW included five presentations of the CS+ and CS-. No US was presented in EXT, RCL, or RNW. Tasks were presented using Presentation Software (Neurobehavioral Systems, Berkley, CA, USA).

Throughout all tasks, the startle response was quantified as the peak-to-peak orbicularis EMG value in the 21-150ms window after probe onset according to (14). All startle responses were visually inspected and scored as missing values if a voluntary blink occurred just before, during, or after probe onset, or if there were any other artifacts obscuring the response. To account for individual differences in startle magnitude, startle responses to the CS+ were

standardized to the mean startle response during ITI/rest trials, calculated as follows: [average startle to CS+]/[average startle during rest], with values > 1 indicating potentiation of the startle response, as previously described (12). To assess the effects of extinction training after acquisition, difference scores were calculated as the standardized response to CS+ at a given phase (e.g. EXT early) – response at ACQ, with values < 1 indicating decreased responding, or extinction of the conditioned response.

Affective Image Task. Affective images were selected from the International Affective Picture System [IAPS; (15)] and divided into four separate sets matched based on normative ratings of valence and arousal. Each task contained 12 images of each category (positive, neutral, negative), matched for the presence or absence of social content, as in (*16*). During the task, participants viewed a single image for 6 sec and then rated valence from -4 (negative) to +4 (positive) and physiological arousal on a scale of 0 to 9. Facial EMG recordings of the zygomatic and corrugator were assessed throughout the task. EMG response to task stimuli were quantified as the mean EMG amplitude during the 6 sec picture presentation compared to the immediately preceding 1 sec baseline. Participants provided self-reported ratings of arousal (0 to 9) and valence (-4, negative, to +4, positive) immediately following each image. Responses were averaged across stimulus type; positive, neutral, and negative images. "Baseline" affective responses were considered to be the responses obtained during the first IAPS task at the first session. The effect of stress on response to affective stimuli was assessed by comparing pre and post stress to control sessions, as previously described (12).

Stress Task. The Maastricht Acute Stress Test (MAST), a modified version of the classic cold pressor task, is a quick and noninvasive approach to elicit robust autonomic and glucocorticoid

stress response (17). The 10 min task consisting of alternating "hand immersion" (HI) trials and "mental arithmetic" (MA) trials. In HI trials, participants placed their left hand in cold water (1-4 degrees Celsius) for up to 90 sec. In MA trials, participants performed mental math aloud, with mistakes resulting in negative feedback (e.g. "start over") and successful attempts resulting in prompting to increase speed. In the control version of the MAST, HI trials were similar but the water was kept at room temperature (20-24 degrees Celsius) and MA trials required participants to count from 1 to 25 at their own pace. Upon completion of each task, participants completed a questionnaire assessing how "unpleasant," "painful," "stressful," and "boring" the task was using a 100mm visual analog scale. In addition, self-reported positive and negative affect before and after the stress and control tasks was assessed via the state version of the Positive and Negative Affect Schedule (PANAS-S). Blood samples were collected via an indwelling catheter in the arm not submerged during the task. Samples were collected immediately before and after the task, as well as after 20 min of recovery. Participants who were unable to have an intravenous catheter successfully inserted or lost patency throughout one or both sessions were eliminated from biochemical analysis.

Biochemical Analysis

Genotyping. DNA was extracted via standard protocols using the InstaGene matrix (Bio-Rad Laboratories, USA) and genotyping at the *FAAH C385A* locus (rs324420) and *CNR1* locus (rs1049353) was done with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, USA) in an Applied Biosystems 7500-Fast Real time PCR (Applied Biosystems, USA) according to manufacturer instructions.

Endocannabinoids. Assay of plasma levels of the endocannabinoids N-arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) and the fatty acid ethanolamides palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) was performed using mass spectrometry. For each sample, 500ul of plasma was directly pipetted into 2ml of acetonitrile with 5 nmol of d8-2-AG, 5 pmol of d8-AEA, 40 pmol d4-PEA, and 40 pmol d4-OEA. Samples were sonicated for 30min in an ice bath and incubated overnight at -20°C to precipitate proteins. The following day samples were centrifuged at 1500xg to remove particulates. The supernatant from each sample was transferred to a new glass tube and evaporated under nitrogen, the tube was then washed once with 350ul acetonitrile (to recapture any lipids adhering to the glass wall) and the acetonitrile was dried under nitrogen gas again. After completely drying, the samples were re-suspended in 200ul of acetonitrile and stored at -80°C until analysis by liquid chromatography mass spectrometry. Analysis in mass spectrometry was performed exactly as previously described (18).

Detection of PF-04457845. The presence of the FAAH inhibitor PF-04457845 from plasma was performed using mass spectrometry and was based on a previously described method (5). First, 500ul of plasma was injected into 2 ml of acetonitrile and samples were sonicated for 30min in an ice bath and incubated overnight at -20°C to precipitate proteins. The following day samples were centrifuged at 1500xg to remove particulates. The supernatant from each sample was transferred to a new glass tube and evaporated under nitrogen, the tube was then washed once with 350ul acetonitrile (to recapture any lipids adhering to the glass wall) and the acetonitrile was dried under nitrogen gas again. After completely drying, the samples were re-suspended in 100ul of 50:50 methanol:water solution and stored at -80°C until analysis by liquid chromatography mass spectrometry.

The LC-MS/MRM analysis was performed using an Eksigent Micro LC200 coupled with an AB Sciex QTRAP 5500 mass spectrometry (AB Sciex, Ontario, Canada). Chromatographic separation of the analytes was carried out on an Eksigent Halo C18 column (2.7 µm, 0.5 × 50 mm, 90Å, AB Sciex). The mobile phase A was 0.1% formic acid in water and the mobile phase B was 0.1% formic acid in acetonitrile. The gradient program was shown as the following: 0-1.2 min (55% B), 1.2-1.7 min (55-95% B), 1.7-3.0 min (95% B), 3-3.5 min (95-55% B), 3.5-5.0 min (55% B). The flow rate is 30 µL/min and the injection volume was 2.0 µL. The data were acquired in positive electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. MRM transitions and collision energy (CE) of all compounds were listed in Table 1. Ion spray voltage was 5500 V. Nebulizer gas (GS 1), auxiliary gas (GS 2), curtain gas (CUR) were 30, 30, 35 (arbitrary units). Collision gas was set as Medium. Declustering potential (DP), entrance potential (EP) and cell exit potential (CXP) were 80, 7 and 14 V.

For quantification, a stock solution of PF-04457845 (1.0 mg/mL) was used to prepare a set of calibrators ranging from 0.1 ng/mL to 50 ng/mL (0.1, 0.5, 1.0, 5.0, 10, 50) with methanol/water (50:50, *v/v*) via a serial dilution. Because the isotopic labelled PF internal standard was not available, concentrations of PF in the samples were determined by an external calibration curve composed by the six calibrators. PF levels were then normalized to quantity per ml of plasma. The lower detection limit was 1 ng/ml and so only samples which registered above this range were quantifiable.

ID	Q1 (Da)	Q3 (Da)	Time (msec)	CE (volt)
PF-1	456	335	500	30
PF-2	456	122	500	30

Supplemental Table S2: Multiple reaction monitoring (MRM) Transition and collision energy (CE) of PF-04457845

Statistical Analysis

Behavioral and biochemical analysis were carried out using one-way or repeated-measures ANOVA (RM-ANOVA) with treatment as a between-subjects factor and an α level of 0.05. If data violated assumptions of normality, Mann-Whitney U-tests were employed. Significant effects were follow-up with Bonferroni post hoc comparisons. Sociodemographic and personality data were evaluated with a one-way analysis of variance (ANOVA) or chi square tests.

For the fear conditioning tasks, the magnitude of the unconditioned startle response was calculated as the average startle response during rest trials and assessed via a one-way ANOVA. Acquisition of fear conditioning was assessed via a RM-ANOVA with cue (CS+, CS-) as a within-subject variable. Fear responding time course through the other task phases (EXT early, EXT late, RCL, RNW) was assessed using a change score ([response to CS+ at ACQ] – [response to CS+ at other task phase]) and assessed using a one-way ANOVA.

Baseline affective responses to emotional stimuli (e.g. responses during the first exposure to the affective image task, regardless of session type) were assessed using RM-ANOVA with stimulus type (positive, neutral, negative) as the within-subject factor for each muscle (corrugator, zygomatic) and self-report rating (valence, arousal) individually. The effect of stress on affective responses was calculated as the change (Post – Pre) in EMG response at each session (stress, control) for each stimulus type (positive, neutral, negative). These change scores were analyzed using a RM-ANOVA with stimulus type as the within-subject factor for each variable (corrugator, zygomatic, valence, and arousal) individually.

Changes in physiological variables (SCR, HR) and subjective stress response were assessed using separate RM-ANOVA with session (stress, control) as a within-subjects factor. Cortisol and eCB response to stress were analyzed using a RM-ANOVA with time (2 time points; pre-

task, post-task recovery) x session (stress, control) as within subjects factors. Baseline differences in serum eCBs and cortisol were analyzed as the dependent variable in a one-way ANOVA.

Supplemental Results

	Placebo N=29	***Drug (confirmed) N=16***	*Drug (total) N=31*
Non-serious adverse events during treatment (10 days)			
Headache	**5**	**3**	3
Cold/respiratory congestion	**2**	**3**	8
Fatigue	**2**	**1**	3
Anxiety	**2**	**1**	5
Sleep difficulties	**2**		
Improved sleep		**1**	1
Nausea	**4**	**1**	2
Cardiovascular		**1**	4
Herpes			1
Joint/muscle pain	**2**		1
Incidence of non-serious adverse events			
Number of participants	**15**	**8**	19
Percentage	**52%**	**50%**	61%

Supplemental Table S3: Non-serious adverse events during treatment. Amount and type of self-reported, non-serious adverse events reported by patients randomized to receive placebo (N = 29) or drug (PF-04457845; N = 31) once a day for ten days. The treatment group is divided into those who were confirmed to receive the drug (confirmed; N = 16), and those who were randomized to the treatment group but did not receive the drug due to a pharmacy error (intended; N = 15; not shown). Incidence of events is calculated as the number of individuals reporting one or more non-serious adverse event throughout the treatment period. No serious adverse events were reported in any group.

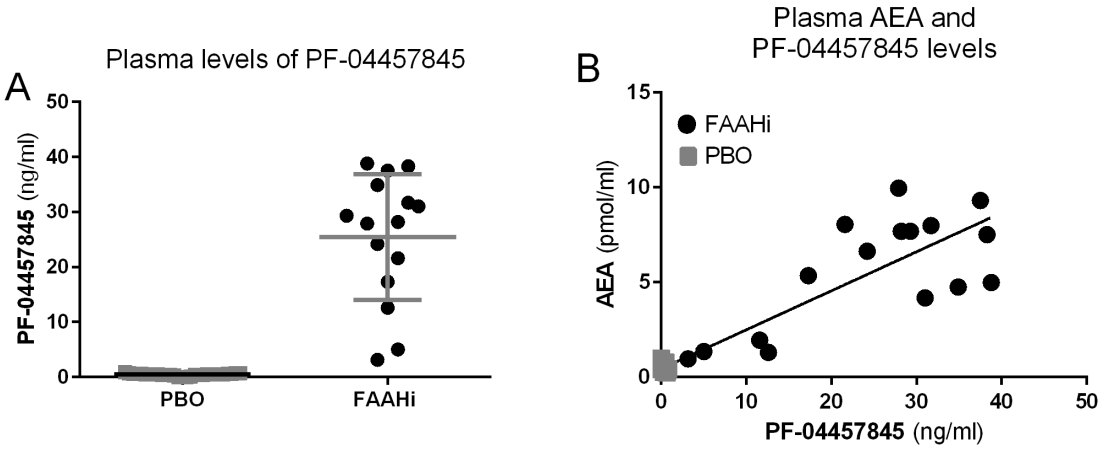
Baseline Participant Characteristics

	Overall (N=45)	***Placebo (N=29)***	***FAAHi (N=16)***	***Test Statistic***
Sex	**N (% total)**	**N (% total)**	**N (% total)**	
Men	19 (42%)	12 (41%)	7 (44%)	$X(1)=0.02$, $p=0.88$
Women	26 (58%)	17 (58%)	9 (56%)	
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	
Age	25.5 (7.2)	26.1 (8.4)	24.6 (4.0)	$F(1,43)=0.45$, $p=0.51$
BMI	23.5 (2.6)	23.1 (2.6)	24.2 (2.7)	$F(1,43)=1.72$, $p=0.20$
AUDIT	4.6 (2.4)	4.0 (2.1)	5.3 (2.6)	$F(1,43)=2.85$, $p=0.10$
DUDIT	0.2 (0.7)	0.2 (0.8)	0.1 (0.5)	$F(1,43)=0.29$; $p=0.60$
STAI-T	35.4 (5.3)	35.9 (4.9)	34.6 (5.9)	$F(1,43)=0.57$; $p=0.46$

Supplemental Table S4: Participant demographics. FAAHi – Fatty acid amide hydrolase inhibitor (PF-04457845); AUDIT - Alcohol Use Disorders Identification Test; DUDIT – Drug Use Disorders Identification Test; BMI – Body Mass Index; STAI-T – Spielberger State-Trait Anxiety Inventory (trait version). Values represent total N and percent of total or mean and standard deviation from the mean.

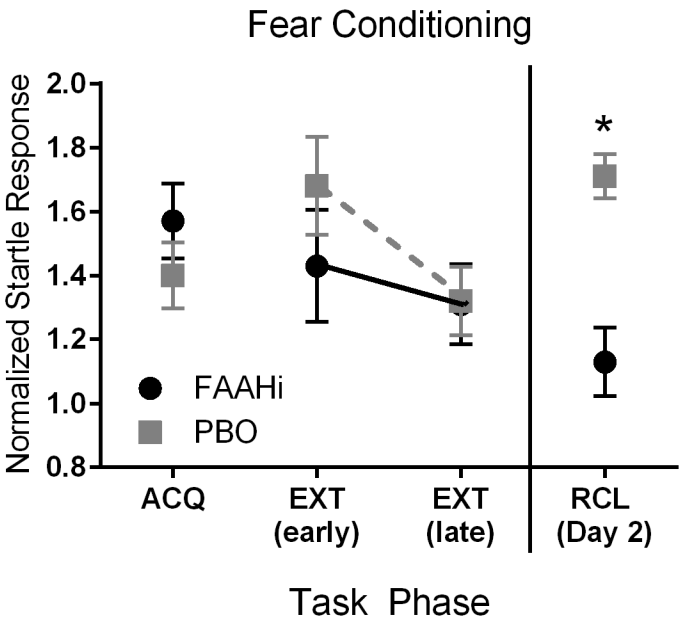
Baseline AEA Is Markedly Elevated by FAAH Inhibition

Plasma drug concentrations were significantly elevated in the FAAHi group (Supplemental Fig S2A) as compared to the placebo group. Moreover, drug levels were significantly correlated with post-treatment levels of AEA (*Spearman's* $\rho=0.66$; $p<0.001$; Supplemental Fig S2B) and OEA (*Spearman's* $\rho=0.68$; $p<0.001$), but not with post-treatment levels of PEA (*Spearman's* $\rho=0.022$; $p=0.89$) or 2-AG (*Spearman's* $\rho=-0.28$; $p=0.068$).



Supplemental Figure S2: Plasma levels of PF-04457845 and AEA. A) Plasma levels of PF-04457845 following 10 days of treatment with placebo (PBO; N = 29) or the FAAH inhibitor PF-04457845 (FAAHi; n = 16); effect of group, $p < 0.001$. B) Correlation between plasma levels of PF-04457845 and AEA; *Spearman's* $\rho = 0.66$, $p < 0.001$.

FAAH Inhibition Does Not Affect Acquisition of Conditioned Fear but Promotes Recall of Fear Extinction



Supplemental Figure S3: Normalized startle responses at each phase of the fear conditioning task. There is no effect of FAAHi on the acquisition of conditioned fear (ACQ) or within-session extinction (EXT). However, the FAAHi group demonstrates enhanced recall of fear extinction (RCL), as shown by a suppression of startle responding to the conditioned stimulus on day 2. Normalized startle responses are calculated as [(average startle response to CS+)/(average response startle response during inter-trial intervals)] for a given task phase with values >1 indicate potentiation of the startle response. Values represent means ± standard error of the mean. FAAHi = fatty acid amid hydrolase inhibitor (PF-04457845); PBO = placebo. * $p < 0.001$ effect of treatment.

Baseline Affect Is Not Affected by FAAH Inhibition

Prior to stress exposure, affective images elicited the expected EMG and self-reported responses, but this was not influenced by FAAH inhibition. Specifically, negative images increased corrugator reactivity while positive pictures reduced it [$F(2,86)=42.4$, $p<0.001$, *partial* $\eta^2=0.50$; Fig 4A]; and positive pictures elicited the greatest zygomatic reactivity [$F(2,86)=15.3$, $p<0.001$, *partial* $\eta^2=0.26$]. We also found expected effects of stimulus type on ratings of valence [$F(2,86)=816$, $p < 0.001$, *partial* $\eta^2 =0.93$] and arousal [$F(2,86)=63.3$, $p<0.001$, *partial* $\eta^2=0.60$]. There was, however, no effect of treatment or treatment*stimulus interaction on any measure of baseline affective response (*corrugator:* type*treatment

p=0.49, treatment *p*=0.79; *zygomatic*: type*treatment *p*=0.78, treatment *p*=0.64; *valence*: type*treatment *p*=0.94, treatment *p*=0.49; *arousal*: type*treatment, *p*=0.94, treatment, *p*=0.49).

Stress-induced Negative Affect Is Attenuated by FAAH Inhibition

There was no effect of stress on any other response to the affective images (for details, see *Supplemental Materials)* including zygomatic reactivity (stimulus type, *p*=0.49; type*treatment *p*=0.73; treatment *p*=0.27), self-reported valence (stimulus type *p*=0.52; type*treatment *p*=0.12; treatment, *p*=0.58), or arousal (stimulus type *p*=0.21, type*treatment *p*=0.14, treatment *p*=0.71).

Stress-induced Decrease in AEA Is Absent Following FAAH Inhibition

FAAH inhibition prevented stress-induced decreases in circulating AEA levels. Specifically, we found a significant effect of treatment on AEA [treatment: $F(1,36)=73.9$, $p<0.001$, *partial* $\eta^2=0.67$; effect of time: $F(1,36)=4.55$, $p=0.040$, *partial* $\eta^2=0.11$; treatment*session*time interaction: $F(1,36)=4.31$, $p=0.045$, *partial* $\eta^2=0.11$; Fig 5D]. Post-hoc tests revealed a significant effect of session (stress, control) in placebo-treated ($p_{uncorrected}=0.009$) but not FAAH inhibitor-treated participants ($p=0.65$).

There was also a significant between-subjects effect of treatment on OEA levels overall [$F(1, 36) = 52.2$, $p < 0.001$, *partial* $\eta^2 = 0.59$], but no effect of session or time [session, $p = 0.13$; time, $p = 0.34$; session*treatment, $p = 0.11$; time*treatment, $p = 0.29$; session*time, $p = 0.42$; session*time* treatment, $p = 0.73$].

For PEA, we found significant session*time [$F(1,36) = 5.29$, $p = 0.27$, *partial* $\eta^2 = 0.13$] and session*time*treatment interactions [$F(1,36) = 5.29$, $p = 0.039$, *partial* $\eta^2 = 0.11$] and a

marginal effect of time [$F(1,36) = 3.95$, $p = 0.054$, *partial* $\eta^2 = 0.10$]. Follow-up tests revealed that stress increased PEA in drug-treated participants ($p_{uncorrected} = 0.034$) but not PBO-treated ($p = 0.81$).

There was no significant effect of stress or treatment on 2-AG [treatment, $F(1,369) = 3.16$, $p = 0.084$, *partial* $\eta^2 = 0.08$; session, $p = 0.37$; time, $p = 0.82$; session*time, $p = 0.50$; session*treatment, $p = 0.71$; time*treatment, $p = 0.47$; session*time*treatment, $p = 0.75$]. Details can be found below in *Supplemental Table S5*.

	Control Session				Stress Session			
	Pre	*Post*	*Difference*	*% Change*	*Pre*	*Post*	*Difference*	*% Change*
AEA								
PBO	0.43 (0.03)	0.53 (0.04)	0.08 (0.04)*	22.9 (3.70)	0.48 (0.03)	0.50 (0.04)	0.02 (0.03)	5.34 (3.38)≠
FAAHi	5.79 (0.83)	5.87 (0.81)	0.01 (0.35)	6.10 (5.64)	5.47 (0.75)	5.81 (0.85)	0.20 (0.20)	7.32 (4.61)
OEA								
PBO	4.82 (0.31)	6.65 (0.88)	0.96 (0.46)*	18.6 (4.00)	5.44 (0.51)	6.19 (0.64)	0.86 (0.25)*	16.5 (4.88)
FAAHi	46.0 (7.17)	46.3 (6.84)	-1.95 (1.57)	2.71 (2.03)	43.4 (7.45)	44.0 (7.18)	-0.37 (1.49)	2.82 (3.41)
PEA								
PBO	61.8 (3.75)	66.5 (5.11)	6.84 (7.50)	24.9 (18.3)	59.6 (5.09)	66.0 (5.51)	6.84 (7.50)	30.9 (15.6)
FAAHi	73.4 (5.39)	68.1 (7.67)	13.8 (8.31)	-2.45 (10.7)	60.6 (4.13)	87.2 (8.84)	26.2 (7.11)*	46.0 (12.0)≠
2-AG								
PBO	8.87 (1.32)	7.73 (0.92)	-1.29 (0.79)	12.0 (20.6)	9.99 (2.33)	9.37 (2.26)	-0.45 (3.06)	23.5 (21.4)
FAAHi	5.98 (0.89)	5.41 (0.71)	-0.57 (0.58)	-2.94 (7.63)	5.34 (0.61)	7.28 (1.97)	1.77 (1.92)	35.5 (29.4)

Supplemental Table S5: Baseline and stress-induced changes in circulating endocannabinoids. Absolute values of AEA, OEA, PEA, and 2-AG before ("pre") and after ("post") control and stress tasks. Difference scores are calculated as [Post – Pre]; percent change from baseline is calculated as [(Post-Pre)/Pre*100]. Values are mean (in pmol/ml) ± standard errors of the mean. % change = percent change from baseline (baseline = "pre"). AEA = anandamide, OEA = oleoylethanolamide; PEA – palmitoylethanolamide;, 2-AG = 2-arachidonylglycerol; *p < 0.05 effect of time; ≠p < 0.05 effect of session.

Results of Entire Study Sample (N = 60)

Results comparing individuals intended and confirmed to receive placebo (N = 29), intended (but not confirmed) to receive FAAH inhibition (N = 15), and those intended and confirmed to receive FAAH inhibition (N = 16). Analyses were carried out using Dunnett's post-hoc test to

compare the placebo group (PBO) to i) the intended (not confirmed) FAAH inhibitor group ($FAAH_{int}$) and the confirmed FAAH inhibitor group ($FAAH_{conf}$). Baseline measures: there was no effect of treatment on self-reported anxiety (STAI-S: $F(2,57) = 0.02$, $p = 0.98$; PBO vs $FAAH_{int}$ $p = 0.98$; PBO vs $FAAH_{conf}$ $p = 1.00$), positive affect (PANAS-Positive: $p = 0.86$; PBO vs $FAAH_{int}$ $p = 0.83$ PBO vs $FAAH_{conf}$ $p = 0.99$), or negative affect (PANAS-Negative: $p = 0.24$; vs $FAAH_{int}$ $p = 0.83$ PBO vs $FAAH_{conf}$ $p = 0.32$).

FAAH Inhibition Increases Anandamide

Presence of drug was confirmed plasma of $FAAH_{conf}$ individuals only (PBO vs $FAAH_{int}$ Mann-Whitney $U = 203$; $p = 0.71$; PBO vs $FAAH_{conf}$ Mann-Whitney $U = 0.00$, $p < 0.001$). We found an effect of treatment such that $FAAH_{conf}$ participants had significantly higher baseline levels of AEA (PBO vs $FAAH_{int}$ Mann-Whitney $U = 206$, $p = 0.78$; PBO vs $FAAH_{conf}$ Mann-Whitney $U = 1.00$, $p < 0.001$) and OEA (PBO vs $FAAH_{int}$ Mann-Whitney $U = 178$ $p = 0.41$; PBO vs $FAAH_{conf}$ Mann-Whitney $U = 1.00$, $p < 0.001$). There was no effect of treatment on PEA ($F(2,56) = 0.07$, $p = 0.93$, *partial* $\eta 2 = 0.002$; PBO vs $FAAH_{int}$ $p = 1.00$; PBO vs $FAAH_{conf}$ $p = 0.94$), 2-AG ($F(2,56) = 0.46$, $p = 0.63$, *partial* $\eta 2 = 0.016$; PBO vs $FAAH_{int}$ = 1.00 ; PBO vs $FAAH_{conf}$ $p = 0.60$), or cortisol ($F(2,59) = 0.21$, $p = 0.81$, *partial* $\eta 2 = 0.008$; PBO vs $FAAH_{int}$ $p = 0.85$; PBO vs $FAAH_{conf}$ $p = 0.97$).

FAAH Inhibition Does Not Affect Acquisition of Conditioned Fear, but Promotes the Recall of Fear Extinction

FAAH inhibition did not influence the acquisition of conditioned fear. There was a main effect of cue type (CS+, CS-) on the start response ($F(1,55) = 25.9$, $p < 0.001$, *partial* $\eta 2 = 0.32$) but this did not differ between groups (cue*group: $p = 0.58$; PBO vs $FAAH_{int}$ $p = 1.00$; PBO vs

$FAAH_{conf}$ p = 0.48). There was no effect of treatment on ratings of US aversiveness (F(2,57) = 0.53, p = 0.59; PBO vs p = 0.51; PBO vs $FAAH_{conf}$ p = 0.87).

There was no effect of treatment on early (F(2,56) = 1.93, p = 0.16, *partial η2* = 0.065; PBO vs $FAAH_{int}$ p = 0.93; PBO vs $FAAH_{conf}$ p = 0.17) or late extinction (F(2,56) = 1.08, p = 0.35; PBO vs $FAAH_{int}$ p = 0.91; PBO vs $FAAH_{conf}$ p = 0.26). FAAH inhibition enhanced the recall of fear extinction (F(2,56) = 6.56, p = 0.003, *partial η2* = 0.19; PBO vs $FAAH_{int}$ p = 0.78; PBO vs $FAAH_{conf}$ p = 0.001) but only in the confirmed FAAH ($FAAH_{conf}$) group. FAAH inhibition did not influence renewal of fear (F(2,56) = 1.24, p = 0.30: PBO vs $FAAH_{int}$ p = 1.00; PBO vs $FAAH_{conf}$ p = 0.27).

FAAH Inhibition Does Not Impact Baseline Affect

At baseline, there was no effect of treatment on corrugator activity (effect of stimulus type: F(2,114) = 45.1, p < 0.001; *partial η2* = 0.45; group*type F(4,114) = 0.45, p = 0.77; PBO vs $FAAH_{int}$ p = 0.81; PBO vs $FAAH_{conf}$ p = 0.98), zygomatic activity (effect of stimulus type: F(2,114) = 20.3, p < 0.001, *partial η2* = 0.26; group*type p = 0.78; PBO vs $FAAH_{int}$ p = 0.62; PBO vs $FAAH_{conf}$ p = 0.86), self-reported valence (effect of stimulus type: F(2,114) = 884, p < 0.001, *partial η2* = 0.94; stimulus*group p = 0.66; PBO vs $FAAH_{int}$ p = 0.64; PBO vs $FAAH_{conf}$ p = 0.44), or self-reported arousal (effect of stimulus type: F(2,114) = 88.2, p < 0.001, *partial η2* = 0.61; stimulus*group p = 0.93; PBO vs $FAAH_{int}$ p = 0.82; PBO vs $FAAH_{conf}$ p = 0.75).

Stress-induced Affect Is Attenuated by FAAH Inhibition

Following stress exposure, there was an interaction between treatment and stimulus type on corrugator activity (effect of stimulus F(2,114) = 1.97, p = 0.14; type*group F(14,114) = 2.81, p = 0.029, *partial η2* = 0.090; PBO vs $FAAH_{int}$ p = 0.99; PBO vs $FAAH_{conf}$ p = 0.087). Post hoc follow up test revealed that confirmed FAAH inhibition reduced corrugator activity in response

to negative stimuli ($F(2,57) = 8.20$, $p = 0.001$; *partial η2* = 0.22; PBO vs $FAAH_{int}$ $p = 0.94$ PBO vs $FAAH_{conf}$ $p = 0.001$), but not neutral ($p = 0.95$; PBO vs $FAAH_{int}$ $p = 0.99$; PBO vs $FAAH_{conf}$ $p = 0.93$) or positive ($p = 0.92$; PBO vs $FAAH_{int}$ $p = 0.90$; PBO vs $FAAH_{conf}$ $p = 1.00$).

There was no effect of treatment on zygomatic activity (effect of stimulus type: $p = 0.64$; stimulus*group $p = 0.88$; PBO vs $FAAH_{int}$ $p = 0.85$; PBO vs $FAAH_{conf}$ $p = 0.43$), self-reported valence (effect of stimulus type: $p = 0.74$; stimulus*group $p = 0.37$; PBO vs $FAAH_{int}$ $p = 0.88$; PBO vs $FAAH_{conf}$ $p = 0.81$) or self-reported arousal (effect of stimulus type: $p = 0.68$; stimulus*group $p = 0.33$; PBO vs $FAAH_{int}$ $p = 0.68$; PBO vs $FAAH_{conf}$ $p = 0.93$).

FAAH Inhibition Attenuates Autonomic, but Not Endocrine Stress Responses

There was an effect of stress (effect of session $F(1,52) = 65.4$, $p < 0.001$, *partial η2* = 0.56) and group*session interaction (session*group $F(2,52) = 3.55$, $p = 0.036$; *partial η2* = 0.12; PBO vs $FAAH_{int}$ $p = 0.98$; PBO vs $FAAH_{conf}$ $p = 0.050$) such that confirmed FAAH inhibition was associated with reduced SCR frequency. Stress increased self-reported ratings of subjective stress (effect of session: $F(1,57) = 361$, $p < 0.001$, *partial η2* = 0.86) and there was a non-significant trend towards an interaction with treatment and session ($F(2,57) = 2.79$, $p = 0.082$, *partial η2* = 0.084; PBO vs $FAAH_{int}$ $p = 0.85$; PBO vs $FAAH_{conf}$ $p = 0.38$). Post hoc follow up tests revealed that FAAH inhibition reduced ratings of “stressful” at the stress session ($F(2,57) = 7.07$, $p = 0.002$, *partial η2* = 0.20; PBO vs $FAAH_{int}$ $p = 0.92$; PBO vs $FAAH_{conf}$ $p = 0.003$) but only in the confirmed FAAH-treated group.

There was a main effect of session ($F(1,52) = 256.0$; $p < 0.001$, *partial η2* = 0.33) and a session*time interaction ($F(1,52) = 46.1$; $p < 0.001$, *partial η2* = 0.47) such that stress increased cortisol levels but this was not influenced by group ($p = 0.46$; PBO vs $FAAH_{int}$ $p = 0.78$; PBO vs $FAAH_{conf}$ $p = 0.65$). Similarly, stress increased heart rate but this did not differ between groups

(effect of session $F(1,55) = 43.8$; $p < 0.001$, *partial η2* = 0.44; session*group $p = 0.92$; PBO vs $FAAH_{int}$ $p = 0.47$; PBO vs $FAAH_{conf}$ $p = 0.71$). Stress increased self-reported negative affect but this was against consistent between groups (PANAS-Negative: $F(1,53) = 48.8$, $p < 0.001$, *partial η2* = 0.48; sess*group $p = 0.60$; PBO vs $FAAH_{int}$ $p = 0.99$; PBO vs $FAAH_{conf}$ $p = 0.72$). Stress did not influence self-reported ratings of positive affect (PANAS-Positive: $p = 0.59$; session*group $p = 0.35$; PBO vs $FAAH_{int}$ $p = 0.50$; PBO vs $FAAH_{conf}$ $p = 0.91$).

Stress-induced Changes in AEA Are Absent Following FAAH Inhibition

In regards to AEA levels, there was a main effect of time ($F(1,49) = 6.33$, $p = 0.015$; *partial η2* = 0.11), treatment ($F(2,49) = 57.8$, $p < 0.001$; *partial η2* = 0.70; PBO vs $FAAH_{int}$ $p = 0.99$; PBO vs $FAAH_{conf}$ $p < 0.001$), and a marginally significant session*time*group interaction ($F(2,49) = 3.04$, $p = 0.057$; *partial η2* = 0.11).

There was a main effect of time ($F(1,49) = 3.38$; $p = 0.072$, *partial η2* = 0.065) and treatment ($F(2,49) = 39.9$; $p < 0.001$, *partial η2* = 0.62) on OEA levels (PBO vs $FAAH_{int}$ $p = 0.99$; PBO vs $FAAH_{conf}$ $p < 0.001$).

There was a trend towards a main effect of time on PEA levels ($F(1,49) = 3.95$; $p = 0.052$, *partial η2* = 0.075), a session*time interaction ($F(1,49) = 5.42$; $p = 0.024$, *partial η2* = 0.10), but no main effect of group ($p = 0.37$; PBO vs $FAAH_{int}$ $p = 0.99$; PBO vs $FAAH_{conf}$ $p = 0.31$).

There were no significant effects on 2-AG (effect of group: PBO vs $FAAH_{int}$ $p = 0.27$; PBO vs $FAAH_{conf}$ $p = 0.10$).

Supplemental References

1. K. Ahn *et al.*, *J. Pharmacol. Exp. Ther.* **338**, 114–124 (2011).
2. J. P. Huggins, T. S. Smart, S. Langman, L. Taylor, T. Young, *Pain*. **153**, 1837–1846 (2012).
3. D. C. D'Souza *et al.*, *The Lancet Psychiatry*. **6**, 35–45 (2019).
4. D. S. Johnson *et al.*, *ACS Med Chem Lett*. **2**, 91–96 (2011).
5. G. L. Li *et al.*, *British journal of clinical pharmacology*. **73**, 706–716 (2012).
6. I. Boileau *et al.*, *Journal of cerebral blood flow and metabolism: official journal of the International Society of Cerebral Blood Flow and Metabolism*. **35**, 1237–40 (2015).
7. A. Mullard, *Nature Reviews Drug Discovery*. **16**, 447 (2017).
8. A. Kerbrat *et al.*, Acute Neurologic Disorder from an Inhibitor of Fatty Acid Amide Hydrolase. *https://doi.org/10.1056/NEJMoa1604221* (2016), doi:10.1056/NEJMoa1604221.
9. A. C. M. van Esbroeck *et al.*, *Science*. **356**, 1084–1087 (2017).
10. A. Persson *et al.*, *Journal of addiction medicine*. **11**, 119–125 (2017).
11. M. R. Milad, S. P. Orr, R. K. Pitman, S. L. Rauch, *Psychophysiology*. **42**, 456–64 (2005).
12. L. M. Mayo *et al.*, *Mol Psychiatry* (2018), doi:10.1038/s41380-018-0215-1.
13. D. L. Neumann, A. M. Waters, *Biol Psychol*. **73**, 175–185 (2006).
14. T. D. Blumenthal *et al.*, *Psychophysiology*. **42**, 1–15 (2005).
15. P. J. Lang, M. K. Greenwald, M. M. Bradley, A. O. Hamm, *Psychophysiology*. **30**, 261–73 (1993).
16. M. C. Wardle, H. de Wit, *Psychopharmacology*. **220**, 143–53 (2012).
17. T. Smeets *et al.*, *Psychoneuroendocrinology*. **37**, 1998–2008 (2012).
18. M. Qi, M. Morena, H. A. Vecchiarelli, M. N. Hill, D. C. Schriemer, *Rapid communications in mass spectrometry : RCM*. **29**, 1889–97 (2015).