Constitutive Increases in Corticotropin-Releasing Factor and Fatty Acid Amide Hydrolase Drive an Anxious Phenotype

Supplemental Information

Supplemental Methods and Materials

General Information

Chemicals. Neurotransmitter, benzoyl chloride, ^{13}C-benzoyl chloride, borate, artificial cerebral spinal fluid (aCSF) and hydroxypropyl β-cyclodextrin (HBC) salts were all purchased from Sigma Aldrich (St. Louis, MO). Hydrochloric acid (HCl), sodium hydroxide (NaOH), and dimethyl sulfoxide (DMSO) solutions were also purchased from Sigma Aldrich. Deuterated products were purchased from Caymen Chemicals (Ann Arbor, MI), except for d4- and $^{13}C_2$-ethanolamine purchased from Cambridge Isotope Laboratories (Andover, MA). HPLC-grade solvents including water (H_2O), acetonitrile (ACN), methanol (MeOH), ammonium formate, and formic acid (FA) were purchased from VWR. Dubelcco's phosphate buffered saline (PBS) was purchased from Thermo Fisher Scientific (Waltham, MA). The selective FAAH inhibitor PF-3845 was purchased from Organix (Woburn, MA). The peptide corticotropin-releasing factor (CRF) human/rat was purchased from Tocris Biosciences (Minneapolis, MN). The fluorophosphonate-rhodamine probe was prepared and provided by Dr. Benjamin Cravatt (1), and the selective CRF_1 antagonist N,N-bis(2-methoxyethyl)-3-(4-methoxy-2-methylphenyl)-2,5-dimethylpyrazolo[1,5-a]pyrimidin-7-amine (MPZP) was prepared and provided by Dr. Kim Janda (2).

Drug Preparation. Drugs were prepared for *in vivo* studies as described: PF-3845 was solubilized in vehicle (1:1:18; ethanol, emulphor, and saline), and administered intraperitoneally (i.p.) at either 3 or 10 mg/kg (1 mL/kg). CRF was solubilized in vehicle (bacteriostatic saline, 1% FA), and administered by intracerebroventricular (i.c.v.) injection (1 μg/2 μL) in a 2-min period. MPZP was acidified (1N HCl, 10% of final volume), diluted in HBC (80% of final volume), backtitrated (1N and 0.1N NaOH) to a final stock solution of 5 mg/mL (20% HBC, pH 4.4), and administered subcutaneously (s.c.) at 2 mL/kg for a cumulative dose of 10 mg/kg. For *ex vivo* slice electrophysiology, CRF (100 nM) was dissolved in H_2O and PF-3845 (1 μM) was dissolved in DMSO. Drugs were administered in tissue sections at a final DMSO concentration of less than 1%.

Specificity of PF-3845. Exposure of PF-3845 (1 μM, 10 min) in *ex vivo* amygdalar slices collected from Wistar rats using similar protocols as the electrophysiological studies demonstrated nearly complete inhibition of the major endocannabinoids (eCB) clearance enzyme fatty acid amide hydrolase (FAAH), with no significant effects on other metabolic enzymes such as monoacylglycerol lipase (MAGL), and α/β-hydrolase domain-containing 6 (ABHD6) or off-target serine hydrolases (see Figure S4A,B). For *in vivo* studies, previously published studies demonstrate that 3-10 mg/kg dosing produces similarly selective effects on brain FAAH (3). To confirm that these doses produce changes in brain eCBs levels, amygdalar tissue from animals treated with PF-3845 (10 mg/kg, i.p., 2h post-treatment) was analyzed for eCB content and demonstrated that this dose resulted in increases in ethanolamide levels of N-arachidonoylethanolamine (anandamide, AEA), palmitoyolethanolamine (PEA), and oleoylethanolamine (OEA) without affecting 2-arachidonoylglycerol (2-AG) (Figure S4C).

Anxiety-like Behavioral Assessments

Elevated Plus Maze (EPM). The testing room contained a dim light that was centered above the maze such that the central platform received about 60 lux, and each of the open arms received about 20 lux of illumination. To reduce extraneous sounds, a 65-dB white noise generator was placed in the back of the room. Rats were habituated to testing conditions for at least 4 days (1h per day), and for at least 1h on test day. On test day, rats were shuttled into a dark anteroom where they received an injection of PF-3845 (3 or 10 mg/kg; ip), vehicle, or no injection and were left undisturbed for 2h prior to testing. Drug groups were tested on separate occasions using identical testing conditions and with appropriate vehicle controls. Rats were individually brought into the testing room, and placed on the central platform whereby an observer noted the duration of time spent and the number of entries made into the open and closed compartments of the apparatus. The percentage of time spent on the open arm of the maze during the 5 min test period was used to index anxiety-like behavior, whereas generalized maze activity was indexed by the total number of closed-arm entries.

Novelty-induced Hypophagia. Prior to training, rats were isolated in their cages with plastic dividers and given overnight access to bottles containing a highly palatable chocolate solution (*Boost Plus*®, Nestle Healthcare Nutrition Inc., Florham Park, NJ). Rats received vehicle injections 30 min into their dark cycle and were left undisturbed for 2h. During training, rats were brought into a quiet testing room illuminated by red light. After a 15 min acclimation, rats were presented with the chocolate solution for 30 min. Following training, rats were transported into the vivarium, and the total volume consumption of the chocolate solution (in mL) was recorded. Training procedures continued for 3 days until stable intake was reached. Cages were left unchanged throughout baseline procedures. On day 4, rats were processed in a similar

manner; however, baseline measures of latency to first drink from the bottle (in sec) as well as total consumption of the chocolate solution were recorded. On day 5, half of the animals from each genotype were injected with PF-3845 (10 mg/kg; i.p.) or vehicle, and processed in a similar manner; however, to induce novelty stress, rats were transferred to new cages with wire flooring, and the room lights and a 65-dB white noise generator were turned on. Similar measures of latencies and total consumption were recorded. Previous studies indicate an anxiety-like effect during novelty stress procedures, resulting in an increase in the latency to approach, and a reduction in the volume consumption of palatable substances (4, 5).

Biochemical Assessments of eCB Clearance Enzymes

Activity-based Protein Profiling (ABPP). ABPP is a chemical proteomics approach that uses small-molecule probes to determine the functional state of enzymes in native systems (6). The fluorophosphonate ABPP probe selectively labels the catalytic serine of active serine hydrolase enzymes (including FAAH, MAGL, and ABHD6), but not their inactive forms, facilitating the characterization of changes in enzyme activity. The specificity of the fluorophosphonate-probe assay for measuring FAAH, MAGL and ABHD6 has been previously determined using overexpression systems (7), knockout animals (8, 9), and/or mass spectrometry (7, 10-12).

FAAH Hydrolysis. Amygdalar proteomes (50 μg/mL, 49 μl volume, PBS) were incubated with d4-AEA substrate (1 μL, in DMSO) at multiple final concentrations (10, 5, 2, 1, 0.2, and 0.1 μM) for 30 min at room temperature. Activity was quenched with HCl (2 μL, 1.2 M) and chilled ACN containing 600 pmol of the internal standards d8-AEA and $^{13}C_2$-ethanolamine (EA). Samples were centrifuged (500 x g, 10 min, 4ºC), aliquoted (10 μL), and supplemented with

equal volumes of mobile phase (25 mM ammonium formate, 75% v/v ACN, 25% H_20, and 0.1% FA). Samples (10 μL, 4ºC) were then isocratically separated by hydrophilic interaction liquid chromatography (HILIC) using a PolyLC Polyhydroxyethyl A column (100 × 1.0 mm, 3 μm, Columbia, MD) at 0.1 ml/min and 30°C, and analyzed by positive-ion mode tandem quadrupole mass spectrometry (Agilent 6460 QQQ, Santa Clara, CA) using multiple-reaction monitoring. D4-AEA and d4-EA were quantified using the standard isotope dilution method at the following transition masses (precursor→product): d4-AEA (352→66), d8-AEA (356→62), d4-EA (66→48), and $^{13}C_2$-EA (64→36). The inverse of turnover values [1/(pmol/min/mg)] were used to calculate the maximum velocity of enzymatic reaction (Vmax) and binding affinity (Km).

Neurochemical Assessments of eCBs and Neurotransmitters

In-vivo Microdialysis of eCBs. Rats were implanted unilaterally with intracerebral guide cannulas (SciPro Inc., Sanborn, NY, MAB 2/6/9.14.IC) aimed 1 mm above the CeA (from bregma AP: -2.3, ML: ±4.0, DV: -6.4, dura). Following recovery, rats were lightly anesthetized and microdialysis probes (SciPro Inc., MAB 6.14, 1 mm PES active region, 15kD MWCO) were inserted and secured into the guide cannulas. The probes were perfused with filtered aCSF composed of the following: (in mM) 149 NaCl, 2.8 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 0.25 ascorbate, and 5.4 D-glucose, pH 7.2–7.4 adjusted with NaOH, and set to circulate at 0.2 μL/min with a syringe pump. Twelve hours later, the perfusate was replaced with aCSF containing 30% w/v HBC as per previously established methods (13), and the flowrate was raised to 0.6 μL/min and allowed to equilibrate for at least 1h. Samples were collected for 1.5h, placed on dry ice, and stored at -80°C. Animals were sacrificed, and brains were extracted for histological assessment of probe placement.

ECB Quantification. Quantification of AEA, 2-AG, PEA and OEA were performed using liquid chromatography coupled with mass spectrometry (LC-MS). Briefly, microdialysate samples (5 µL) were spiked with an internal standard solution (5 µL) containing 25 nM of the following deuterated internal standards: d4-AEA, d5-2-AG, d4-OEA and d4-PEA. Samples (8 µL, 4°C) were injected for analysis by LC-MS (Agilent 1200 MSD). Metabolites were analyzed in positive-ion mode using the following [M+Na+] ions: AEA (+370 m/z), d4-AEA (+374 m/z), PEA (+322 m/z), d4-PEA (+326 m/z), OEA (+348 m/z), d4-OEA (+352 m/z), 2-AG (+401 m/z), d5-2-AG (+406 m/z). Concentrations were calculated using the standard isotope dilution method. Sample concentrations were expressed as an absolute value (nM).

In-vivo Microdialysis of Neurotransmitters. Rats were prepared with guide cannulas (PlasticsOne, C312GA/SPC 21GA) into the CeA as mentioned above. Following recovery, rats were lightly anesthetized and homemade microdialysis probes were inserted and secured into the guide cannulas. Probes were constructed as previously described (14). Briefly, a 300 µm o.d. regenerated cellulose dialysis membrane (Spectrum Laboratories, Inc.; Laguna Beach, CA; 13kD MWCO) was fitted over two lines of silica tubing (Polymicro Technology, Phoenix, AZ; 40 µm i.d., 105 µm o.d.) held in a shaft of stainless steel tubing (McMasters-Carr; Los Angeles, CA; 26GA Thin Wall). We followed similar preparations for dialysis collection; however, the perfusate remained as clean aCSF. Baseline samples were collected for 1h, and then rats received an injection of PF-3845 (10 mg/kg; ip) or vehicle and were left undisturbed for 2h. Following pretreatment, samples were collected for 1h, and then all animals were subjected to restraint stress for 30 min. Additional samples were collected for 1h after the termination of restraint stress procedures. Samples were placed on dry ice, and stored at -80°C until neurochemical

analysis. Animals were sacrificed, and brains were extracted for histological assessment of probe placement.

Neurotransmitter Quantification. Quantification of neurotransmitters was performed using triple quadruple mass spectrometry (LC-MS/MS) methods as previously established (15, 16). Briefly, microdialysate samples (5 µL) were derivatized with 100 mM borate (2.5 µL) and 2% benzoyl chloride (2.5 µL, in ACN), and subsequently spiked with ^{13}C-labeled internal standards (2.5 µl, in 98% v/v of DMSO, 1% FA, and 1% H2O). Samples (10 µl, 4°C) were separated by high performance liquid chromatography and analyzed by positive-ion mode tandem quadrupole mass spectrometry (Agilent 6460 QQQ) using multiple-reaction monitoring. The following neurotransmitters were quantified using the standard isotope dilution method (precursor→product): the amino acids aspartate (238→105), gamma-aminobutyric acid (GABA, 208→105), glutamate (252→105), glutamine (251→105), glycine (180→105), serine (210→105), taurine (230→105), and the monoamines dopamine (466→105), norepinephrine (482→105) and serotonin (385→264). Baseline concentrations were expressed as an absolute value (nM), while changes produced by restraint were expressed either as relative values (% of baseline) or area under the curve (AUC).

Electrophysiological Recordings

Intracellular Recording of Evoked Glutamatergic Responses. We recorded evoked excitatory postsynaptic potentials (eEPSPs) from 48 medial CeA neurons (n = 24 from 13 msPs, n = 24 from 14 Wistars) with sharp micropipettes filled with 3M KCl using discontinuous current-clamp by stimulating the adjacent basolateral amygdala through a bipolar stimulating electrode. We held neurons near their resting membrane potential [Wistar: -77.4±1.0 (n=22),

msP: -81.4±0.9 mV (n=23)]. Average input resistance by genotype was 157.1±9.5 MΩ for Wistar and 166.1±11.1 MΩ for msP. Data were acquired with an Axoclamp-2A preamplifier and stored for later analysis using pClamp software (Axon Instruments, Foster City, CA). We pharmacologically isolated eEPSPs by using the GABA receptor blockers bicuculline (30 μM) and CGP55845A (1 μM). Following most of the recordings, we superfused 30 μM DNQX and 30 μM DL-AP-5 to confirm the glutamatergic nature of the eEPSPs. To determine the synaptic response parameters for each cell, we performed an input-output (I/O) protocol consisting of a range of five current stimulations, beginning at the threshold current required to elicit an EPSP up to the strength required to elicit the maximum amplitude. The stimuli strengths were maintained during the entire experiment. We examined paired-pulse facilitation (PPF) in each neuron using paired stimuli at 50 and 100 msec inter-stimulus interval. The stimulus strength was adjusted such that the amplitude of the first eEPSP was 50% of maximal, determined from the I/O relationship. We calculated the PPF ratio as the second EPSP amplitude over that of the first EPSP. We then quantified the synaptic responses by calculating the EPSP amplitude with Clampfit 10.2 (Molecular Devices).

Table S1. *Amygdalar binding affinities for FAAH hydrolysis are comparable across genotype.* Values reflect mean ± SEM binding affinity (Km) in amygdalar tissue of non-selected Wistar and msP rats treated with the corticotropin-releasing factor type 1 (CRF_1) receptor blocker N,N-bis(2-methoxyethyl)-3-(4-methoxy-2-methylphenyl)-2,5-dimethylpyrazolo[1,5-a]pyrimidin-7-amine (MPZP, 10 mg/kg, 2 mL/kg, s.c., b.i.d., n=7 per genotype) or vehicle (n=8 per genotype). There were no main effects of drug treatment ($F_{(1,26)}$=1.8, N.S.), genotype ($F_{(1,26)}$=3.3, N.S.), or genotype x drug interactions ($F_{(1,26)}$=1.5, N.S.).

Km Binding Affinity (∝M ± SEM)	Wistar	msP
Vehicle	0.93 ± 0.06	1.58 ± 0.39
MPZP	0.90 ± 0.04	1.02 ± 0.06

Table S2. *ANOVA statistics for the FAAH hydrolysis studies.* Values reflect additional statistics performed for the studies examining the substrate conversion of deuterated N-arachidonoylethanolamine (d4-AEA) into ethanolamine (d4-EA) in amygdalar tissue. In the MPZP study, non-selected Wistar and msP rats were treated with the corticotropin-releasing factor type 1 (CRF_1) receptor blocker N,N-bis(2-methoxyethyl)-3-(4-methoxy-2-methylphenyl)-2,5-dimethylpyrazolo[1,5-a]pyrimidin-7-amine (MPZP, 10 mg/kg, 2 mL/kg, s.c., b.i.d., n=7 per genotype) or vehicle (n=8 per genotype). In the CRF study, Wistar rats received administration of the peptide hormone CRF (1 µg/2 µL, i.c.v., n=7) or vehicle (n=8). Plus sign (+) denotes a significant increase relative to vehicle controls ($p<0.05$).

FAAH Hydrolysis	d4-Anandamide						
MPZP Study	[d4-AEA]	0.1 ∝M	0.2 ∝M	1 ∝M	2 ∝M	5 ∝M	10 ∝M
	Genotype	$F_{(1,26)}$=0.6, N.S.	$F_{(1,26)}$=1.1, N.S.	$F_{(1,26)}$=0.02, N.S.	$F_{(1,26)}$=0.2, N.S.	$F_{(1,26)}$=0.8, N.S.	$F_{(1,26)}$=1.2, N.S.
	Drug	$F_{(1,26)}$=0.1, N.S.	$F_{(1,26)}$=0.4, N.S.	$F_{(1,26)}$=0.2, N.S.	$F_{(1,26)}$=0.2, N.S.	$F_{(1,26)}$=0.01, N.S.	$F_{(1,26)}$=0.3, N.S.
	Genotype x Drug	$F_{(1,26)}$=0.2, N.S.	$F_{(1,26)}$=1.0, N.S.	$F_{(1,26)}$=0.4, N.S.	$F_{(1,26)}$=0.8, N.S.	$F_{(1,26)}$=0.1, N.S.	$F_{(1,26)}$=0.3, N.S.
CRF Study	Drug	$F_{(1,13)}$=2.8, N.S.	$F_{(1,13)}$=3.8, p=0.074	$F_{(1,13)}$=4.5, p=0.053	$F_{(1,13)}$=7.2, **+**p<0.05	$F_{(1,13)}$=4.0, p=0.068	$F_{(1,13)}$=3.2, N.S.

Table S3. *Elevated plus maze (EPM) performance in msP versus Wistar rats.* Values reflect mean ± SEM of time spent in the arms of the EPM, number of arm entries, and percentage of time and entries relative to totals in non-selected Wistar and msP rats (n=5-7 per genotype). Asterisks (*) denote significant genotype differences ($p<0.05$).

Elevated Plus Maze	Wistar		msP
Time in Arm (sec)	Open	88.6 ± 25.7	25.0 ± 11.2*
	Closed	165.4 ± 27.6	263.0 ± 12.6*
Number of Arm Entries	Open	6.6 ± 1.7	1.6 ± 0.7*
	Closed	9.2 ± 2.1	4.0 ± 1.1*
	Total	15.8 ± 3.3	5.6 ± 1.6*
Percentage of Total Time	Open	35.1 ± 9.9	8.8 ± 3.9*
	Closed	64.9 ± 9.9	91.2 ± 3.9*
Percentage of Total Entries	Open	39.7 ± 7.4	23.6 ± 8.9
	Closed	60.3 ± 7.4	76.4 ± 8.9

Table S4. *Elevated plus maze (EPM) performance in msP versus Wistar rats following PF-3845 administration.* Values reflect mean ± SEM of time spent in the arms of the EPM, number of arm entries, and percentage of time and entries relative to totals in non-selected Wistar and msP rats pretreated with doses of the fatty acid amide hydrolase (FAAH) inhibitor PF-3845 (3 or 10 mg/kg, i.p., n=12-15 per dose and genotype) or vehicle (n=19-20 per genotype). The following effects were significant: Open arm time: genotype x drug interaction ($F_{(2,83)}$=3.6, p<0.05); Closed arm time: genotype x drug interaction ($F_{(2,83)}$=3.3, p<0.05); Open arm entries: genotype main effect ($F_{(1,83)}$=16.9, p<0.001); Closed arm entries: genotype main effect ($F_{(1,83)}$=29.3, p<0.001); Total entries: genotype main effect ($F_{(1,83)}$=39.3, p<0.001); Percent open arm time: genotype x drug interaction ($F_{(2,83)}$=3.2, p<0.05); Percent closed arm time: genotype x drug interaction ($F_{(2,83)}$=3.2, p<0.05). Asterisks (*) denote significant genotype differences, whereas plus signs (+) denote significant differences relative to vehicle controls (p<0.05). A symbol shown on "Open", "Closed", or "Total" denotes significant main effects.

Elevated Plus Maze	Wistar				msP		
Time in Arm (sec)	PF-3845	Vehicle	3 mg/kg	10 mg/kg	Vehicle	3 mg/kg	10 mg/kg
	Open	83.8 ± 10.1	63.0 ± 15.5	69.7 ± 7.3	30.2 ± 5.2*	40.0 ± 5.7	64.5 ± 13.0+
	Closed	135.3 ± 7.9	159.2 ± 13.9	143.2 ± 9.5	205.1 ± 8.7*	182.7 ± 7.0	170.4 ± 15.3+
Number of Arm Entries	Open*	5.7 ± 0.6	5.4 ± 1.2	4.8 ± 0.5	2.4 ± 0.4	3.7 ± 0.5	3.6 ± 0.5
	Closed*	10.3 ± 0.6	9.5 ± 0.9	9.4 ± 0.9	6.6 ± 0.6	7.5 ± 0.4	5.9 ± 0.6
	Total*	16.1 ± 0.9	14.8 ± 1.7	14.2 ± 1.1	9.0 ± 0.8	11.2 ± 0.7	9.4 ± 0.6
Percentage of Total Time	Open	37.3 ± 4.1	27.6 ± 6.6	33.1 ± 3.5	13.2 ± 2.3*	17.9 ± 2.4	27.9 ± 5.7+
	Closed	62.7 ± 4.1	72.4 ± 6.6	66.9 ± 3.5	86.8 ± 2.3*	82.1 ± 2.4	72.1 ± 5.7+
Percentage of Total Entries	Open	35.5 ± 2.7	32.8 ± 5.3	34.9 ± 3.1	24.4 ± 3.3	31.9 ± 3.4	37.3 ± 4.6
	Closed	64.5 ± 2.7	67.2 ± 5.3	65.1 ± 3.1	75.6 ± 3.3	68.1 ± 3.4	62.7 ± 4.6

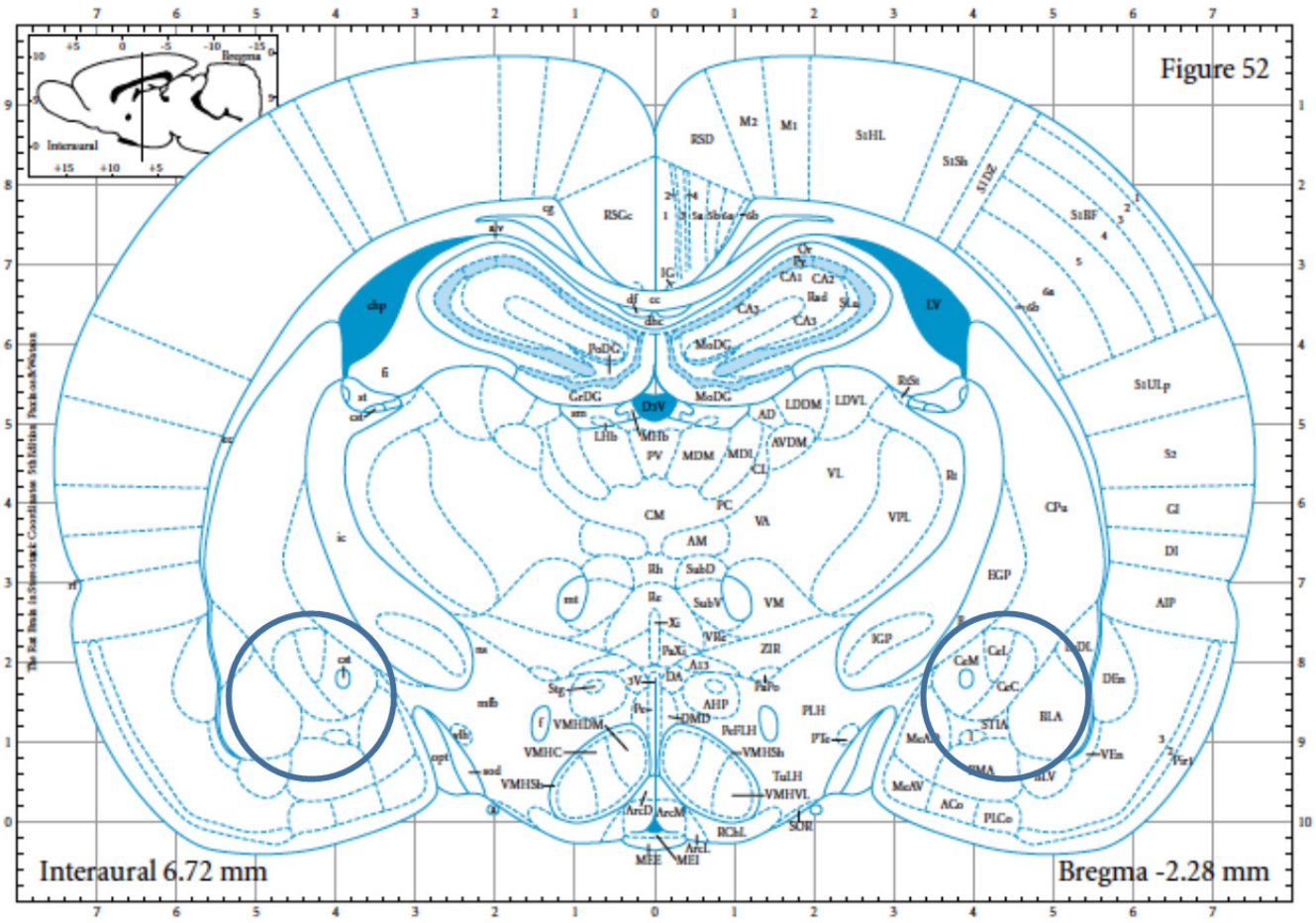

Figure S1. *Rat amygdalar dissection for biochemical assays*. Approximate wet tissue dissections of a 2 mm amygdalar slice using a 12-gauge tissue extractor aimed at the ventral portion between the internal and external capsule (17).

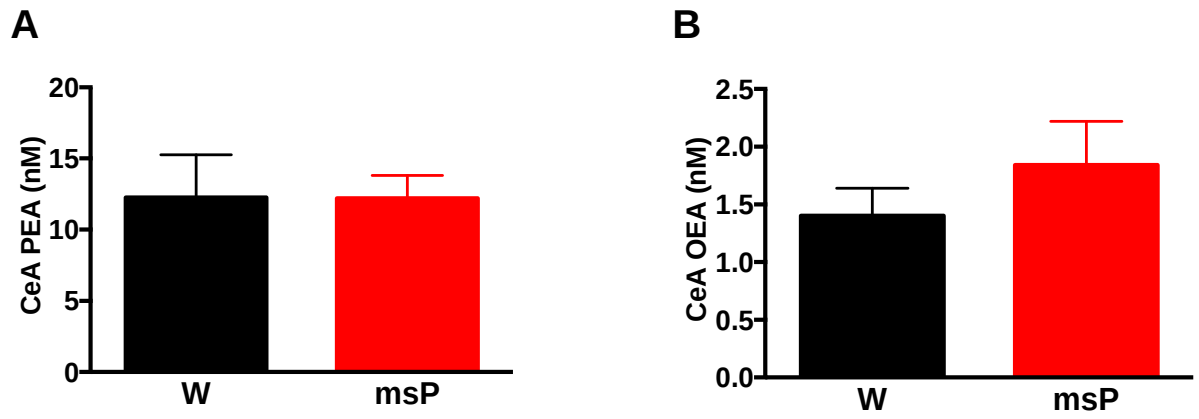

Figure S2. *Dialysate concentrations of the ethanolamides PEA and OEA in the central amygdala are comparable across genotype.* (**A**) Baseline dialysate concentrations of palmitoyol-ethanolamine (PEA) in the central nucleus of the amygdala (CeA) of non-selected Wistar (n=9) and msP (n=10) rats. (**B**) Baseline dialysate concentrations of oleoylethanolamine (OEA) in the CeA of rats from **A**. Data expressed as mean ± SEM. There were no significant differences in dialysate levels of PEA ($F_{(1,17)}$=0.02, N.S.) or OEA ($F_{(1,17)}$=1.0, N.S.).

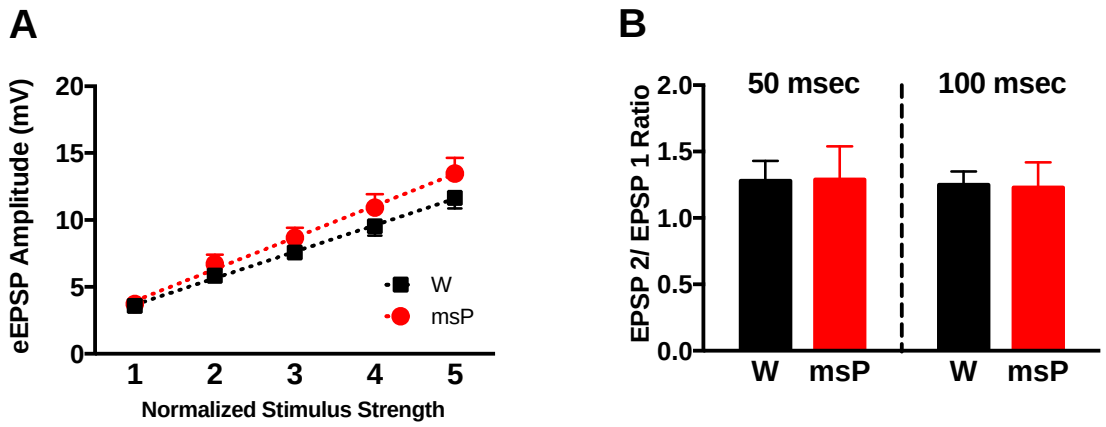

Figure S3. *Baseline evoked glutamatergic excitatory postsynaptic potentials in the central amygdala are comparable across genotype.* (**A**) Baseline input-output (I/O) curves of excitatory postsynaptic potentials (EPSPs) evoked by basolateral amygdala (BLA) stimulation (beginning at the threshold current required to elicit an EPSP up to the strength required to elicit the maximum amplitude) in neurons from the central nucleus of the amygdala (CeA) of non-selected Wistar (W, n=24 neurons from 13 rats) and msP (n=24 neurons from 14 rats) rats. (**B**) Histograms plotting the baseline paired-pulse facilitation (PPF) ratios at 50 and 100 msec in CeA neurons of rats from **A**. Data expressed as mean ± SEM. There were no genotypic differences in baseline eEPSP I/O curves ($F_{(1,45)}$=1.6, N.S.) or PPF ratios ($F_{(1,51)}$=0.01, N.S.).

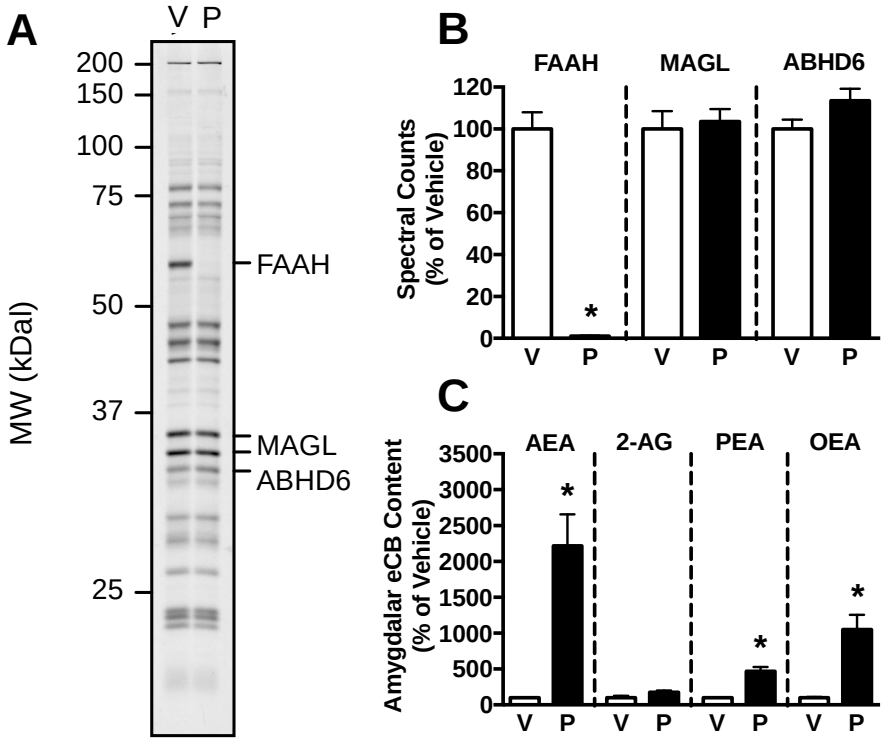

Figure S4. *PF-3845 selectively inhibits FAAH and increases amygdalar anandamide content.* (**A**) Representative gel image of serine hydrolase activity following incubation of the selective inhibitor of fatty acid amide hydrolase (FAAH) PF-3845 (P, 1 μM) or vehicle (V) in tissue sections of the central nucleus of the amygdala collected from non-selected Wistar (n = 4) rats. (**B**) Percentage of spectral counts in active site labeling of the endocannabinoid clearance enzymes FAAH, monoacylglycerol lipase (MAGL), and α/β-hydrolase domain containing 6 (ABHD6) in rats from **A**. (**C**) Percentage change in amygdalar tissue content of N-arachidonoylethanolamine (AEA), 2-arachidonoylglycerol (2-AG), and the ethanolamides palmitoyolethanolamine (PEA) and oleoylethanolamine (OEA) in Wistar rats receiving PF-3845 administration (10 mg/kg; i.p.; n = 4) or vehicle (n = 3). Data expressed as mean ± SEM. Asterisks (*) denote significant differences relative to vehicle controls ($p<0.05$).

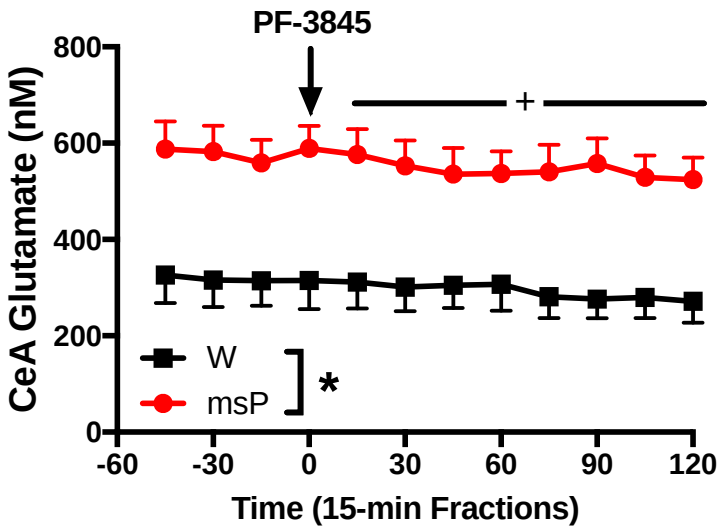

Figure S5. *FAAH inhibition mildly reduces glutamate levels in the rat central amygdala.* Dialysate levels of glutamate in the central nucleus of the amygdala (CeA) across time in which PF-3845 (10 mg/kg, i.p.) was administered (arrow) in non-selected Wistar (n = 9) and msP (n = 9) rats. Data expressed as mean ± SEM. Significant main effects are denoted by a continuous line with asterisks (*) for genotype differences ($F_{(1,16)}$=13.6, p<0.01) and plus signs (+) for time effects ($F_{(11,176)}$=3.4, p<0.001). There was no genotype x time interaction ($F_{(11,176)}$=0.55, N.S.).

Supplemental References

1. Patricelli MP, Giang DK, Stamp LM, Burbaum JJ (2001): Direct visualization of serine hydrolase activities in complex proteomes using fluorescent active site-directed probes. *Proteomics*. 1:1067-1071.
2. Richardson HN, Zhao Y, Fekete EM, Funk CK, Wirsching P, Janda KD, et al. (2008): MPZP: a novel small molecule corticotropin-releasing factor type 1 receptor (CRF1) antagonist. *Pharmacol Biochem Behav*. 88:497-510.
3. Ahn K, Johnson DS, Mileni M, Beidler D, Long JZ, McKinney MK, et al. (2009): Discovery and characterization of a highly selective FAAH inhibitor that reduces inflammatory pain. *Chem Biol*. 16:411-420.
4. Bechtholt AJ, Hill TE, Lucki I (2007): Anxiolytic effect of serotonin depletion in the novelty-induced hypophagia test. *Psychopharmacology (Berl)*. 190:531-540.
5. Bluett RJ, Gamble-George JC, Hermanson DJ, Hartley ND, Marnett LJ, Patel S (2014): Central anandamide deficiency predicts stress-induced anxiety: behavioral reversal through endocannabinoid augmentation. *Transl Psychiatry*. 4:e408.
6. Galmozzi A, Dominguez E, Cravatt BF, Saez E (2014): Application of activity-based protein profiling to study enzyme function in adipocytes. *Methods Enzymol*. 538:151-169.
7. Blankman JL, Simon GM, Cravatt BF (2007): A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chem Biol*. 14:1347-1356.
8. Alexander JP, Cravatt BF (2005): Mechanism of carbamate inactivation of FAAH: implications for the design of covalent inhibitors and in vivo functional probes for enzymes. *Chem Biol*. 12:1179-1187.
9. Navia-Paldanius D, Aaltonen N, Lehtonen M, Savinainen JR, Taschler U, Radner FP, et al. (2015): Increased tonic cannabinoid CB1R activity and brain region-specific desensitization of CB1R Gi/o signaling axis in mice with global genetic knockout of monoacylglycerol lipase. *Eur J Pharm Sci*. 77:180-188.
10. Hsu KL, Tsuboi K, Chang JW, Whitby LR, Speers AE, Pugh H, et al. (2013): Discovery and optimization of piperidyl-1,2,3-triazole ureas as potent, selective, and in vivo-active inhibitors of alpha/beta-hydrolase domain containing 6 (ABHD6). *J Med Chem*. 56:8270-8279.
11. Hsu KL, Tsuboi K, Speers AE, Brown SJ, Spicer T, Fernandez-Vega V, et al. (2010): Optimization and characterization of triazole urea inhibitors for abhydrolase domain containing protein 6 (ABHD6). *Probe Reports from the NIH Molecular Libraries Program*. Bethesda (MD).
12. Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, et al. (2009): Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat Chem Biol*. 5:37-44.
13. Caille S, Alvarez-Jaimes L, Polis I, Stouffer DG, Parsons LH (2007): Specific alterations of extracellular endocannabinoid levels in the nucleus accumbens by ethanol, heroin, and cocaine self-administration. *J Neurosci*. 27:3695-3702.

14. Parsons LH, Smith AD, Justice JB, Jr. (1991): The in vivo microdialysis recovery of dopamine is altered independently of basal level by 6-hydroxydopamine lesions to the nucleus accumbens. *J Neurosci Methods*. 40:139-147.

15. Song P, Mabrouk OS, Hershey ND, Kennedy RT (2012): In vivo neurochemical monitoring using benzoyl chloride derivatization and liquid chromatography-mass spectrometry. *Anal Chem*. 84:412-419.

16. Buczynski MW, Polis IY, Parsons LH (2013): The volitional nature of nicotine exposure alters anandamide and oleoylethanolamide levels in the ventral tegmental area. *Neuropsychopharmacology*. 38:574-584.

17. Paxinos G, Watson C (2005): *The Rat Brain in Stereotaxic Coordinates*. Elsevier Academic Press.