Endocannabinoid Modulation of Predator Stress-Induced Long-Term Anxiety in Rats

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Abstract

Individuals who experience life-threatening psychological trauma are at risk of developing a series of chronic neuropsychiatric pathologies that include generalized anxiety, depression and drug addiction. The endocannabinoid system has been implicated in the modulation of these responses by regulating the activity of the amygdala and the hypothalamic-pituitary-adrenal axis. However, the role of this signaling complex in the consequences of traumatic events is unclear. Here, we use an animal model of predatory stress-induced anxiety-like behavior to investigate the role of the endocannabinoid system in long-term anxiety. Our main finding is that rats exposed to the red fox pheromone 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), a life-threatening stimulus for these rodents, display a marked increase in the mobilization of the endocannabinoid, 2-arachidonoyl-*sn*-glycerol (2-AG), which is selective to the amygdala. This effect lasts for at least 14 days after the stress has occurred. Additionally, pharmacological inhibition of monoacylglycerol lipase (MGL) – a lipid hydrolase that degrades 2-AG in presynaptic nerve terminals – elevates 2-AG levels in the amygdala and suppresses the anxiety-like behavior elicited by exposure to TMT. The results suggest that predator threat triggers long-term changes in 2-AG-mediated endocannabinoid signaling in the amygdala, and that pharmacological interventions targeting MGL might provide a therapeutic strategy for the treatment of chronic brain disorders initiated by trauma.

Introduction

Traumatic life events heighten the risk of developing neuropsychiatric pathologies such as post-traumatic stress disorder (PTSD), depression, anxiety and substance abuse (Adshead, 2007). In addition to being confronted with the intrusive re-experiencing of trauma-related memories, subjects with PTSD exhibit persistent anxiety states that significantly impair their quality of life (Brewin *et al*, 2000; Gmitrowicz and Kucharska, 1994; Kessler *et al*, 2005; Speckens *et al*, 2006). Our current understanding on the neurobiology of anxiety provides a theoretical basis for the use of pharmacotherapy in the secondary prevention of chronic anxiety after life-threatening events (Davidson, 2004, 2006; Pitman and Delahanty, 2005). A growing body of evidence suggests that pharmacological agents such as β-adrenergic antagonists, opiates, D-cycloserine and anxiolytics are at least partially effective in managing the symptoms of PTSD (Ducrocq and Vaiva, 2005; Zatzick and Roy-Byrne, 2003; Zatzick and Roy-Byrne, 2006; Zhang and Davidson, 2007). However, few studies have thoroughly examined the mechanisms of action and efficacy of psychotherapeutic drugs to prevent persistent anxiety states that develop in the aftermath of major trauma (Adshead, 2007; Davidson, 2004, 2006; Giuffrida *et al*, 2004; Pitman *et al*, 2005).

The endocannabinoid system is a signaling complex that consists of G-protein-coupled receptors, cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2), endogenous lipid-based ligands (mainly anandamide and 2-arachidonoyl-*sn*-glycerol, 2-AG), and proteins involved in the formation and deactivation of such ligands (Kano *et al*, 2009; Piomelli, 2003, 2014). Substantial evidence implicates the endocannabinoid system in the regulation of stress-coping responses (Bortolato *et al*, 2006; Gaetani *et al*, 2003; Hill *et al*, 2010; Kathuria *et al*, 2003; Lutz, 2009; Marsicano *et al*, 2002; Patel and Hillard, 2006, 2008; Steiner *et al*, 2008). Animal experiments suggest that activation of the endocannabinoid system contributes to stress-induced analgesia (Hohmann *et al*, 2005) and enhances stress-coping behaviors in mice and rats (Bortolato *et al*, 2007; Gobbi *et al*, 2005). Additional evidence indicates that anandamide produced in the amygdala modulates the response to acute stressful events

(Gaetani *et al*, 2003; Gunduz-Cinar *et al*, 2013; Marsicano *et al*, 2002). Furthermore, exposure to an auditory fear-conditioning paradigm increases anandamide levels in the basolateral amygdala (BLA) of mice (Marsicano *et al*, 2002) and pharmacological inhibition of the anandamide-degrading enzyme, fatty acid amide hydrolase (FAAH), heightens stress-coping behaviors and exerts anxiolytic-like and anti-depressant-like effects in rodents through a CB_1-dependent mechanism (Gunduz-Cinar *et al*, 2013; Hill *et al*, 2006; Kathuria *et al*, 2003).

Multiple studies have documented the existence of a link between the endocannabinoid system and the hypothalamic-pituitary-adrenal (HPA) axis. Stressful stimuli activate neural inputs to the paraventricular nucleus (PVN) of the hypothalamus, inducing the release of corticotropin-releasing hormone (CRH), which is then transported to the anterior pituitary gland and stimulates the release of adrenocorticotropic hormone (ACTH). Circulating ACTH initiates the release of cortisol (corticosterone in rats) from the adrenal cortex (Pecoraro *et al*, 2006). Interestingly, disruption of CB_1 receptors in the BLA increases HPA-axis activity in non-stressed animals, elevating the plasma concentrations of corticosterone (Hill *et al*, 2010). Accordingly, local administration of a FAAH inhibitor in the BLA suppresses stress-induced activation of the HPA-axis in a CB_1-dependent manner (Hill *et al*, 2009). In addition, a recent human positron emission tomography study on PTSD patients revealed a significant up-regulation of CB_1 receptors within the amygdala-hippocampal-corticostriatal neural circuit, associated with abnormally low levels of circulating anandamide (Neumeister *et al*, 2013). These data suggest that traumatic events can activate endocannabinoid signaling in the brain, which may act as an intrinsic modulator of the response to such events. Indeed, FAAH inhibitors are currently under investigation as potential treatment for anxiety disorders and secondary prevention for PTSD (Finn, 2010). In addition to anandamide, changes in 2-AG have also been implicated in the regulation of stress-induced responses (Evanson *et al*, 2010; Hill *et al*, 2011; Sciolino *et al*, 2011; Sutt *et al*, 2008). However, a systematic investigation of the long-term impact of trauma-induced stress on 2-AG-mediated signaling is still lacking.

In the current study, we show that an acute life-threatening stress, i.e. exposure of rats to predator odor, selectively heightens 2-AG-mediated signaling in the amygdala, but not in other regions of the brain. We further show that systemic or intra-amygdalar inhibition of monoacylglycerol lipase (MGL) activity suppresses anxiety-like behavior triggered by acute exposure to the red fox pheromone 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), an effect prevented by administration of a CB_1 receptor antagonist. The results suggest that pharmacological strategies aimed at enhancing 2-AG signaling at CB_1 receptors may offer a novel therapeutic approach to the treatment of pathological sequelae of psychological trauma, such as PTSD, addiction, anxiety and depression.

Materials and Methods

Animals

We purchased male Sprague-Dawley rats (8-9 weeks, 225-250 g) from Charles River (Wilmington, MA) and housed them in groups of 3 per cage. We also used an in-house bred colony of C57BL6J mice (25-30 g). The animals were maintained on a 12 h light/dark cycle (6:30 AM to 6:30 PM) and received food (2020X, Harlan, Madison, WI) and water ad libitum. Animals undergoing surgery were individually housed and acclimated to laboratory conditions for one week prior to surgery. All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Chemicals

We purchased TMT from Contech Enterprises (Delta, B.C., Canada) and butyric acid from Aldrich (St. Louis, MO). JZL184 was either purchased from Cayman Chemical (Ann Arbor, MI) or provided by the National Institute on Drug Abuse (NIDA). Rimonabant was supplied by NIDA

and NF1819 was synthesized in the laboratory of G. Campiani.

Drug administration

We dissolved rimonabant in a vehicle of propylene glycol/Tween-80/sterile saline (0.9%) (1/1/18, vol/vol) and JZL184 in polyethylene glycol-400/Tween-80 (4/1). NF1819 was dissolved in 100% DMSO for intraperitoneal (i.p.) injections and dimethyl sulfoxide (DMSO)/sterile saline (1/39 v/v) for intracerebral infusions. We administered drugs and their vehicle by i.p. injection in a volume of 1 ml/kg.

Surgery

We anesthetized the rats with ketamine (100 mg/kg, i.p., Phoenix Manufacturing Inc., Phoenix, AZ) and xylazine (10 mg/kg; Western Medical Supply, Arcadia, CA), and placed them in a stereotaxic frame (Kopf Instruments, Tujunga, CA) with the nose bar maintained at -3.3 mm relative to the interaural line. In some experiments, we implanted two stainless-steel guide cannulae (7.5 mm; 23 gauge) bilaterally with the tips aimed at the BLA (2.8 mm posterior and 5.0 mm lateral to bregma and 6.2 mm from the skull surface). In other experiments, we placed a single-guide cannula (7.5 mm; 23 gauge) with the tip aimed at the third ventricle (2.3 mm posterior to bregma and 6.5 mm from the skull surface). The cannulae were affixed to the skull with three anchoring screws and dental cement. Dummy cannulae (7.5 mm) were used to maintain patency. The coordinates were histologically verified by taking the flash frozen brain and mounting 20 µm sections on slides and performing a cresyl violet stain (Sigma-Aldrich, St. Louis, MO). After surgery, the rats were individually housed and allowed to recover for 8 days before training and handling.

Intracerebral infusion

We dissolved NF1819 in a vehicle of dimethyl sulfoxide (DMSO)/sterile saline (1/39 v/v). We

infused individual doses of the drug into the BLA (5 ng, 0.2 µL) or the third ventricle (75 ng, 3 µL) using a 10-µL Hamilton syringe and a 30-gauge injector (Plastics One, Chicago, IL) connected to the cannula via polyethylene tubing. The infusion needle extended 2 mm beyond the end of the guide cannula. An automated pump PHD 2000 (Harvard, Holliston, MA) drove the syringe at the rate of 0.1µL/min. The infusion needles were left in place for an additional 30 s to allow the solution to diffuse. Immediately following the procedure, the animals were returned to their home cages.

Brain tissue preparation

We excised brain regions of interest by taking micropunches from frozen brains mounted on a Microm HM525 cryostat (Thermo Fisher Scientific, Waltham, MA). The following brain regions were selected (coordinates, in mm from bregma)(Paxinos and Watson, 1998): hypothalamus, single 2 mm×4 mm punch (from −0.8 to −4.8); amygdala, bilateral 2 mm×2 mm punch (from −1.6 mm to −3.6); dorsal hippocampus, bilateral 2 mm×2 mm punch (from −1.6 mm to −3.6); ventral hippocampus, bilateral 1.5mm×2mm punch (from −4.16 mm to −6.16); cerebellum, single 2 mm×2 mm punch (from −10.3 mm to −11.3 mm). The punches were transferred to 8-mL glass vials on dry ice and kept frozen at −80 °C until time of processing.

Lipid analyses

We homogenized brain tissue samples (7-15 mg) in methanol (1 ml) containing deuterium-labeled 2-AG (0.5 nmol) and anandamide (10 pmol) as internal standards (Cayman, Ann Arbor, MI). We extracted lipids with chloroform (2 ml) and water (1 ml). The organic phases were dried under N_2, reconstituted in chloroform (2 ml) and fractionated by open-bed silica gel column chromatography as described (Fu *et al*, 2007). The eluted fractions containing 2-AG and anandamide were dried under N_2, and residues were suspended in chloroform/methanol (1/3, vol/vol; 60 µL). Analyses were conducted using a liquid chromatography (LC) apparatus

consisting of an Agilent 1100 system and 1946D mass spectrometer (MS) detector equipped with electrospray ionization interface (Agilent Technologies, Santa Clara, CA, USA). Anandamide and 2-AG were separated on a ZORBAX Eclipse XDB-C18 column (2.1 x100 mm, 1.8 µm, Agilent Technologies) using an acetonitrile gradient. Solvent A consisted of water containing 0.1% formic acid, and Solvent B consisted of acetonitrile containing 0.1 % formic acid. We used the following gradient profile: 0-15 min, 65% B; 15-16 min, 65-100% B linear gradient; 16-26 min, 100% B; 26-28 min, 100-65% B linear gradient; 28-30 min, 65% B. The column temperature was kept at 15°C and the flow rate at 0.3 ml/min. ESI was in the positive ionization mode, capillary voltage was set at 3 kV, and the fragmentor voltage was set at 70 V. Nitrogen gas was used as a drying gas at a flow rate of 13 liters/min and a temperature of 350°C. The nebulizer pressure was set at 40 psi. Absolute amounts of 2-AG and anandamide were quantified using a calibration curve.

MGL activity assay

We homogenized frozen brain samples in 10 volumes of ice-cold Tris-HCl (50 mM, pH 7.5) containing sucrose (0.32 M), centrifuged the homogenates at 1,000×g for 10 min, and incubated the supernatants (0.1 mg of protein) at 37°C for 30 min in Tris-HCl (0.5 mL) containing heptadecenoylglycerol (10 µM; NuCheck Prep, Elysian, MN) as substrate. Reactions were stopped by adding 1.5 mL of chloroform-methanol (2/1) containing heptadecanoic acid (NuCheck Prep) as internal standard. After centrifugation at 1,000×g at 4°C for 10 min, we collected the organic layers, dried them under N_2, suspended the residues in chloroform/methanol (1/3, 60 µL) and analyzed them by LC/MS (see above).

Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative PCR

We extracted RNA from brain punches using a TRIzol (Invitrogen)/ RNeasy (QIAGEN) hybrid protocol. First-strand complementary DNAs were synthesized from 1 µg of the total RNA using

SuperScript II RNase H reverse transcriptase (Invitrogen) and oligo-dT12–18 primers, for 50 min at 42°C. Quantitative PCR was conducted using Mx3000P system (Stratagene) by a TaqMan method. All mRNA levels were normalized using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal standard. The primer/probe set were as follows: for rat c-fos forward, 5'-TTCCGGCATCATCTAGGCC-3'; reverse, 5'-ACAGGTCCACATCTGGCACA-3'; TaqMan probe, 5'-AGTGGCTCGGAGACTGCCCGC-3'; for rat CB_1 receptor forward, 5'-CAAGCACGCCAACAACACAG-3'; reverse, 5'- TCTTAACGGTGCTCTTGATGCA-3'; TaqMan probe, 5'-TGCACAGGGCCGCGGAGAG-3'. for rat diacylglycerol lipase-α (DGL-α) forward, 5'-CCAGGCCTTTGGGCG-3'; reverse, 5'-GCCTACCACAATCAGGCCAT-3'; TaqMan probe, 5'-ACCTGGGCCGTGGAACCAAACA-3'; for rat MGL forward, 5'-CCCAGTGGCACACCCAAG-3'; reverse, 5'-TAACGGCCACAGTGTTCCC-3'; TaqMan probe, 5'-CCCTCATCTTCGTGTCCCATGGAGC-3'. We used TaqMan gene expression assays for rat GAPDH (Rn01775763_g1) (Life Technologies, Carlsbad, CA).

Odor Exposure

For 5 consecutive days, we handled each rat for 2 min and placed it for 10 min in a plastic exposure box (45 x 25 x 20 cm) containing a square of gauze (5 x 5 cm) doused with saline (35 µl). The box was housed in a fume hood. On the day of the experiment, we randomly assigned the animals to the saline or TMT group. The rats were placed for 10 min in the exposure box containing a gauze doused either with saline or TMT (35 µl). After the procedure, the animals were immediately returned to their home cages.

Elevated plus maze

We conducted the behavioral tests between 8:00 AM and 2:00 PM. The elevated plus maze (EPM) apparatus included two open (50×10 cm) and two closed (50×10×40 cm) arms extending from a central platform (10 ×10 cm) elevated 60 cm above the floor. We placed each rat in the

central platform of the maze, facing an open arm opposite to the experimenter, and videotaped test sessions of 5-min duration for each trial. Observers blinded to treatment measured the amount of time spent in the open arm, the number of open-arm entries, and the anxiety index. The latter was calculated as 1-(average of percent time in open arm and percent open arm entry). Between tests the apparatus was cleaned with a 20% ethanol solution and was allowed to dry thoroughly. The open arms of the maze were illuminated at 150-170 lux, and the closed arms at 30-40 lux.

Experimental design

To determine the time-course of the effects of TMT exposure, rats were habituated to the setting, exposed to saline or TMT, and then subjected to the EPM test 24 h, 7 days, or 14 days following odor exposure. Each animal was subjected to the test only once. The animals were sacrificed 3 h after the test and their brains were snap-frozen. In a separate experiment, the rats were habituated to the experimental setting, exposed to saline or TMT, and then sacrificed at various time points (30 min, 1 h, 24 h, 7 days, 14 days) following odor exposure. The brains were removed and snap-frozen. To test the effect of systemic MGL inhibition on TMT-induced long-term fear, the rats were habituated to the experimental setting and in addition to the handling procedure, received daily injections of vehicle (4:1 PEG-400:Tween-80, 1 ml/kg, i.p) for 5 days. Eighteen h after odor exposure, vehicle or JZL184 (16 mg/kg, i.p) was injected. Six h or 6 days after JZL184 injections, the animals were subjected to the EPM test. In a separate group of rats, we administered the CB_1 antagonist/inverse agonist, rimonabant (1 mg/kg, i.p.), 30 min before JZL184 and performed an EPM test 6 days later. The animals were sacrificed 3 h after the test and their brains were snap-frozen. Regions of interest were collected from frozen brains and analyzed as described above. Lastly, we investigated the effect of MGL inhibition within the BLA or the hypothalamus. The rats were habituated to the experimental setting as outlined above. Eighteen h after odor exposure, vehicle or NF1819 was infused into the BLA and the EPM test

was performed 6 days later. The animals were sacrificed 3 h after the test and their brains were snap-frozen for analyses.

Statistical analyses

All results are presented as mean±s.e.m. Data were analyzed by two-way analysis of variance (ANOVA). Post-hoc comparisons, when appropriate, were performed by Tukey's multiple comparisons test. In all cases, differences with a $P<0.05$ were considered significant. For all data an extreme Studentized deviate method with $\alpha=0.05$ was performed to identify significant outliers and removed from statistical analysis. TMT-resistant vs TMT-sensitive animal groups were parsed out by calculating the k-means for each cluster and determining the center point.

Results

TMT causes long-term anxiety-like behavior in rats

We challenged male Sprague-Dawley rats with a single 10-min exposure to the fox pheromone TMT (Fendt *et al*, 2005) and measured anxiety-like behavior in the EPM at various time points after odor exposure (24 h, 7 days and 14 days). Compared to saline-exposed animals, rats challenged with TMT exhibited a statistically detectable increase in anxiety-like behavior, which lasted for the entire duration of the experiment (14 days; Figure 1a-c). Exposure to TMT reduced the amount of time spent in the open arms of the maze (TMT effect, $F_{1,42}=16.93$, $p=0.0002$; Figure 1a) and the number of open arm entries (TMT effect, $F_{1,42}=22.67$, $p<0.0001$; Figure 1b), and increased the anxiety index (TMT effect, $F_{1,42}=21.17$ $p<0.0001$; Figure 1c). To verify the behavioral selectivity of the effect of TMT, we challenged a separate group of rats for 10 min with butyric acid, whose odor is pungent but non-threatening to rats. In contrast to TMT, exposure to butyric acid failed to induce anxiety-like responses in the EPM, 24 h after odor presentation (anxiety index: one-way ANOVA, $F_{2,20}=13.68$, $P=0.0002$; Table S1).

Exposure to TMT mobilizes 2-AG in the amygdala

To test whether exposure to TMT affects endocannabinoid signaling in the brain, we challenged rats with the odor and sacrificed them 3 h, 24 h, 7 days, and 14 days later. Of the seven brain regions surveyed (Table S2), two showed statistically detectable changes in 2-AG content, compared to saline-exposed controls: the amygdala, where 2-AG levels increased 24 h after TMT exposure and remained significantly elevated for the following 13 days (TMT effect $F_{1,40}$=34.95 p<0.0001; time after odor $F_{3,40}$=7.19 p=0.0006; Figure 1d); and the hypothalamus, where a transient increase in 2-AG (3 h after TMT) was followed by a short-lasting decrease (24 h after TMT) (interaction $F_{1,40}$=3.443, p=0.026; Figure 1e). In addition to the 2-AG changes, mRNA analyses indicate an increase in c-Fos mRNA levels in the amygdala 24 h after TMT exposure, which returned to baseline levels 6 days later (time after odor, $F_{1,23}$=4.82 p=0.038; interaction, $F_{1,23}$=7.33 p=0.013; Figure 1f). We did not observe significant changes in the transcription of CB_1 receptors, (Figure 1g), MGL, and diacylglycerol lipase-α (DGL-α), a key enzyme of 2-AG production (Figure S1a-f). No change in 2-AG mobilization was detected in the other brain regions included in the survey (Table S2). Additionally, anandamide levels in those regions were not affected by TMT under the present experimental conditions (Table S3). Confirming the selectivity of the response to TMT, exposure to butyric acid had no effect on either 2-AG or anandamide levels (Table S4).

Consistent with published data (Fendt *et al*, 2005; Holman *et al*, 2014), we found that approximately 25% of the animals exposed to TMT did not develop long-term anxiety-like behavior. This lead us to hypothesize that TMT exposure might differentially regulate 2-AG levels in resistant versus sensitive rats. We applied the k-means cluster analysis to the “ratio of time spent in the open arm” data from TMT-exposed rats and determined the center of the clusters (TMT-sensitive vs. TMT-resistant) to be 14.3%. Using this percentage as a threshold, we separated TMT-resistant versus TMT-sensitive rats. As expected, the parsed-out group of TMT-sensitive rats showed high level of anxiety-like behavior relative to TMT-sensitive and

saline-treated rats (one-way ANOVA, $F_{2,21}$=10.59, p=0.0007; Figure 1h), which was associated with elevated 2-AG levels in the amygdala (Figure 1i). Compared to TMT-sensitive rats, TMT-resistant rats had 2-AG levels that were similar to those measured in saline-exposed control animals (one-way ANOVA, $F_{2,21}$=9.29, p=0.0013; Figure 1i).

MGL inhibition prevents TMT-induced long-term anxiety-like behavior

To examine the functional role of 2-AG mobilization following TMT exposure, we interrupted 2-AG degradation *in vivo* using the MGL inhibitor, JZL184 (Pan *et al*, 2009). A single injection of JZL184 (16 mg/kg, i.p.) decreased MGL activity (JZL184 effect, $F_{1,20}$=23.58 p<0.0001; Figure 2a) and increased 2-AG levels (JZL184 effect, $F_{1,20}$=11.46, p=0.0029; Figure 2b) in the rat brain, irrespective of whether the animals were exposed to saline or TMT. As previously reported for other models of anxiety (Sciolino *et al*, 2011), treatment with the MGL inhibitor resulted in a reduction in anxiety-like behavior. Administration of JZL184 (16 mg/kg, i.p.) 18 h after TMT and 6 h before the tests normalized anxiety-like responses in TMT-exposed, but not saline-exposed rats (TMT effect, $F_{1,30}$=14.72 p=0.0006; JZL184 effect, $F_{1,30}$=9.89 p=0.0037; interaction, $F_{1,30}$=10.18 p=0.0033; Figure 2c).

Next we tested whether an early intervention with the MGL inhibitor might influence the development of long-lasting TMT-induced anxiety-like responses. We administered JZL184 (i.p.) 18 h after TMT exposure followed by behavioral tests 6 days later. We found that JZL184 reduced anxiety-like responses in TMT-exposed, but not saline-exposed rats (TMT effect, $F_{1,42}$=11.66 p=0.0014; JZL184 effect, $F_{1,42}$=5.61 p=0.023; interaction, $F_{1,42}$=6.138 p=0.017; Figure 2d). As expected, MGL activity (JZL184 effect, $F_{1,20}$=0.34, p=0.57; Figure 2e) and 2-AG levels (JZL184 effect, $F_{1,20}$=0.28, p=0.6; Figure 2f) were at basal levels 6 days after administration of the drug. The results suggest that temporary enhancement of 2-AG-mediated signaling is sufficient to prevent long-term behavioral changes evoked by predator stress.

The anxiolytic-like effect of JZL184 is CB_1 receptor-dependent

Next we examined whether the long-term anxiolytic-like effects of JZL184 are due to 2-AG-mediated activation of CB_1 receptors. First, we tested whether the CB_1 inverse agonist, rimonabant, has a direct effect on TMT-induced anxiety-like behavior. We administered the drug (1 mg/kg, i.p.) 17.5 h after TMT exposure and tested the behavior after 6 days. The treatment had no effect on the time spent in the open arm (TMT effect $F_{1,18}$=9.24, p=0.007; rimonanbant effect $F_{1,18}$=0.022, p=0.88; Figure 3a), the number of open arm entries (TMT effect $F_{1,18}$=4.88, p=0.04, rimonanbant effect $F_{1,18}$=0.077, p=0.78; Figure 3b), or the anxiety index (TMT effect, $F_{1,18}$=7.87, p=0.012, rimonanbant effect, $F_{1,18}$=0.054, p=0.82; Figure 3c) of animals exposed either to saline or TMT. We then administered rimonabant (1mg/kg, i.p.) 17.5 h after TMT exposure (all rats were exposed to TMT), which was followed by JZL184 injection after 30 min and behavior test after 6 days. Rimonabant suppressed the anxiolytic-like effects of JZL184 (ratio time open arm: interaction, $F_{1,43}$=4.85, p=0.033; ratio entry open arm: interaction, $F_{1,43}$=6.13, p=0.017; anxiety index: interaction, $F_{1,43}$=6.85 p=0.012; Figure 3d-f), providing evidence that that such effect is CB_1-dependent.

MGL inhibition in the BLA blocks TMT-induced anxiety-like behavior

To further explore the mechanism underlying the anxiolytic-like effect of MGL inhibition, we examined whether the amygdala or the hypothalamus, in each of which 2-AG levels change in response to TMT, is responsible for the suppression of TMT-induced anxiety-like behavior. Because of the insoluble nature of JZL184, we used a newly disclosed compound, NF1819, which is a potent, selective and water-soluble MGL inhibitor. The synthesis and general properties of this agent will be reported elsewhere.

To test the efficacy of NF1819, we first administered the drug systemically to adult male mice (5 mg/kg, i.p.). We found that NF1819 markedly reduces MGL activity (p<0.0001) and increases 2-AG content (p<0.0001) in brain tissue within one h of administration (Figure 4a, b). We then

infused NF1819 either into the BLA or the third ventricle of rats (Figure 4c, f). Compared to vehicle, MGL activity was decreased and 2-AG levels were increased in BLA micropunches (MGL activity: p=0.045; 2-AG levels: p=0.0007; Figure 4d, e; micropunch A) but not in micropunches taken that were immediately adjacent to the site of infusion (Figure 4d, e; micropunch B, C). Similarly, infusion of NF1819 into the third ventricle resulted in significant changes in both MGL activity and 2-AG accumulation (MGL activity: p=0.027; 2-AG levels: p=0.0008; Figure 4g, h).

Having confirmed that NF1819 effectively inhibits MGL activity and accrues 2-AG availability after intracerebral administration, we targeted the BLA or the hypothalamus for behavioral testing. We first exposed all the rats to TMT and then infused NF1819 into the BLA 18 h after TMT exposure and 6 days before the behavioral tests. NF1819 reduced anxiety-like responses in rats, compared to vehicle (p=0.0045 Figure 5a). In contrast, infusion of NF1819 into the third ventricle 18 h after TMT exposure and 6 days before the behavioral tests had no significant changes in EPM behavior (p=0.82; Figure 5d-f). The results suggest that the anxiolytic-like effects of MGL blockade are mediated through 2-AG signaling in the BLA.

Discussion

In this study, we report that the long-term anxiety-like state caused in rats by exposure to the fox pheromone TMT is paralleled by a sustained elevation in amygdalar levels of the endocannabinoid 2-AG. We further show that pharmacological inhibition of the 2-AG-hydrolyzing enzyme, MGL, increases 2-AG accumulation and produces marked anxiolytic-like effects, which are abrogated by the CB_1 cannabinoid receptor antagonist, rimonabant. The amygdala is critical to this response, because local infusion of an MGL inhibitor in this region, recapitulates the actions of systemic MGL blockade. Lastly, inhibiting MGL within 24 h of exposure to TMT completely prevents the development of long-term anxiety. The results suggest that 2-AG mobilization in the amygdala acts as an intrinsic feedback mechanism that protects rats against

the chronic consequences of TMT-evoked stress.

An extensive body of research has established that stressful sensory information across multiple modalities enters the amygdala through the BLA and the centrolateral amygdala (CeAL), both of which project to the centromedial amygdala (CeAM) (LeDoux, 2000). The CeAM, in turn, projects to rostral and caudal brain regions involved in the expression of stress, fear, and anxiety (LeDoux, 2000). Functional studies have shown that direct activation of CeAL strongly inhibits CeAM output neurons through GABA(gamma-aminobutyric)-ergic projections and reduces fear and anxiety responses, while direct activation of BLA increases fear and anxiety responses through glutamatergic projections to the CeAM (Etkin *et al*, 2009; Li *et al*, 2013). Because CB_1 receptors expression in the amygdala is restricted to the BLA (Ramikie and Patel, 2012), it is tempting to hypothesize that the persistent increase in amygdalar 2-AG mobilization after TMT exposure provides a negative feedback signal that inhibits the BLA and attenuates CeAM activation. Identifying the precise neural mechanism underlying persistent 2-AG mobilization is an important question that needs to be addressed by future studies.

Previous reports indicate that anandamide plays a protective role in the response to environmental stressors (Hill *et al*, 2013; Hill *et al*, 2011; Kathuria *et al*, 2003; Patel *et al*, 2006) and that stressful stimuli enhance anandamide mobilization in brain regions such as BLA and prefrontal cortex (Bortolato *et al*, 2006; Gaetani *et al*, 2003; Kathuria *et al*, 2003; Lutz, 2009; Marsicano *et al*, 2002; Patel *et al*, 2006, 2008; Steiner *et al*, 2008). In the auditory fear-conditioning paradigm, administration of FAAH inhibitors into the BLA enhances fear extinction and impairs fear memory retrieval (Gunduz-Cinar *et al*, 2013; Marsicano and Lutz, 2006; Marsicano *et al*, 2002). How do anandamide and 2-AG cooperate to modulate the response to stress? While the available data do not allow us to answer this question, it is interesting to note that previous studies have reported a temporal disconnect between the mobilization of anandamide and that of 2-AG. For example, foot shock induces a rapid and short-lived increase

in 2-AG content in the dorsal midbrain of rats, which is accompanied by a slower, more sustained increase in anandamide levels (Hohmann *et al*, 2005). The lack of changes in anandamide levels observed in the present study, compared to those previously documented (Hill *et al*, 2009; Pecoraro *et al*, 2006), might be explained by differences in the time course of mobilization of the two endocannabinoid transmitters in the amygdala. Relevant to this point, a recent study has shown that acute activation of muscarinic acetylcholine receptors in the amygdala causes a short-lived form of anandamide-mediated synaptic depression, while prolonged activation (>1 h) of the same receptor causes a tonic 2-AG-mediated depression (Ramikie *et al*, 2014). Based on these data, it is tempting to speculate that short-term changes in anandamide mobilization play a role in acute stress-coping responses, whereas 2-AG might be involved in the sustained response to a stressor.

The anxiolytic-like effects elicited by MGL inhibitors, JZL184 and NF1819, and their sensitivity to rimonabant, suggest that endogenously produced 2-AG is involved in the regulation of TMT-induced long-term anxiety through the activation of CB_1 receptors in the BLA. These findings complement a growing body of evidence suggesting a role for 2-AG in the modulation of emotional responses to stress (Evanson *et al*, 2010; Hill *et al*, 2011; Sciolino *et al*, 2011; Sutt *et al*, 2008). For example, studies have shown that cat odor exposure causes rapid changes in the expression of enzymes involved in the biosynthesis and degradation of 2-AG in various rat brain regions, including amygdala and periaqueductal grey area, immediately after odor exposure (Sutt *et al*, 2008). These studies did not examine the long-term consequences of odor exposure, which were the object of the present investigation. Additionally, it has been reported that systemic administration of JZL184 reduces anxiety-like behaviors in rats in a CB_1-dependent manner, but only when the animals are exposed to high-stress conditions (i.e., strong environmental light) (Sciolino *et al*, 2011). In agreement with this evidence, the current work suggests that 2-AG acts as an endogenous modulator of anxiety-like behavior, and that this

action might be particularly relevant during conditions of high stress.

Exposure to TMT evokes a prolonged anxiety-like state that is paralleled by an increase in 2-AG levels within the amygdala. A single intra-BLA administration of NF1819 18 h after TMT-exposure evoked anxiolytic-like effects 7 days after the exposure. This suggests that prolonged mobilization of endogenous 2-AG may protect rats against an inappropriately severe long-term anxiety state. The persistent increase in 2-AG content after TMT exposure may be a consequence of acute increase in neuronal activity in specific sets of synapses feeding into the BLA from higher brain regions involved in the stress response. Consistent with this view, we observed a significant increase in amygdalar c-Fos mRNA expression 24 h after TMT-exposure. We hypothesize that MGL in the BLA could modulate neurotransmission between specific synapses engaged in 2-AG signaling, thus destabilizing essential connections and generating long-term anxiety. Conversely, it is also possible that synapses that are not engaged in 2-AG mediated signaling might be responsible for triggering the long-term anxiety-like state in rats, and the 2-AG engaged synapses prevent over activation of this circuit. Therefore, elevated 2-AG content in unengaged synapses may destabilize and silence all communications from the BLA. This is plausible since other studies show that increase of endocannabinoid activity can drive CB_1-dependent synaptic depression in the amygdala (Gunduz-Cinar *et al*, 2013; Ramikie *et al*, 2014). Further investigations are necessary to determine the molecular mechanism(s) of 2-AG mobilization and its relation to long-term anxiety responses. Overall, the 2-AG increase in the BLA may serve as a modulator of anxiety-like response and disruption of this signaling system might result in abnormal response to stress.

Not all people respond to a traumatic event in the same manner (Adshead, 2007; Davidson, 2004, 2006; Giuffrida *et al*, 2004; Pitman *et al*, 2005). Studying individual differences that influence post trauma adaptation may generate clues as to how anxiety states are triggered. Consistent with published data, about 25% of TMT-exposed animals in our study failed to

develop long-term anxiety-like behavior in the EPM (Fendt *et al*, 2005; Holman *et al*, 2014). Additionally, TMT-resistant animals did not exhibit elevated levels of 2-AG in the amygdala compared to TMT-sensitive animals. This suggests that the persistent increase in 2-AG content in TMT-sensitive animals may serve as a natural coping mechanism to an overwhelming stressor, thus also serving as a marker for long-term anxiety. For the TMT-resistant animals, the TMT exposure alone may not have been adequate enough to trigger a significant fear to activate a 2-AG-mediated coping response. Further investigation of the difference in endocannabinoid regulation between TMT-resistant and TMT-sensitive rats should provide valuable insights into the mechanisms underlying the induction of long-term anxiety states.

In conclusion, our study suggests that exposure to predator odor initiates changes in amygdalar 2-AG signaling, which might play a protective role in the response to a traumatic event. The results further suggest that pharmacological agents that enhance 2-AG signaling may attenuate anxiety-like responses in stressed animals and anxiety symptoms in human trauma victims.

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References

Adshead G, & Ferris, S. (2007). Treatment of victims of trauma. *Advances in Psychiatric Treatment* **13**(5): 358-368.

Bortolato M, Campolongo P, Mangieri RA, Scattoni ML, Frau R, Trezza V*, et al* (2006). Anxiolytic-like properties of the anandamide transport inhibitor AM404. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **31**(12): 2652-2659.

Bortolato M, Mangieri RA, Fu J, Kim JH, Arguello O, Duranti A*, et al* (2007). Antidepressant-like activity of the fatty acid amide hydrolase inhibitor URB597 in a rat model of chronic mild stress. *Biological psychiatry* **62**(10): 1103-1110.

Brewin CR, Andrews B, Valentine JD (2000). Meta-analysis of risk factors for posttraumatic stress disorder in trauma-exposed adults. *Journal of consulting and clinical psychology* **68**(5): 748-766.

Davidson JR (2004). Long-term treatment and prevention of posttraumatic stress disorder. *The Journal of clinical psychiatry* **65 Suppl 1**: 44-48.

Davidson JR (2006). Pharmacologic treatment of acute and chronic stress following trauma: 2006. *The Journal of clinical psychiatry* **67 Suppl 2**: 34-39.

Ducrocq F, Vaiva G (2005). [From the biology of trauma to secondary preventive pharmalogical measures for post-traumatic stress disorders]. *L'Encephale* **31**(2): 212-226.

Etkin A, Prater KE, Schatzberg AF, Menon V, Greicius MD (2009). Disrupted amygdalar subregion functional connectivity and evidence of a compensatory network in generalized anxiety disorder. *Arch Gen Psychiatry* **66**(12): 1361-1372.

Evanson NK, Tasker JG, Hill MN, Hillard CJ, Herman JP (2010). Fast feedback inhibition of the HPA axis by glucocorticoids is mediated by endocannabinoid signaling. *Endocrinology* **151**(10): 4811-4819.

Fendt M, Endres T, Lowry CA, Apfelbach R, McGregor IS (2005). TMT-induced autonomic and behavioral changes and the neural basis of its processing. *Neuroscience and biobehavioral reviews* **29**(8): 1145-1156.

Finn DP (2010). Endocannabinoid-mediated modulation of stress responses: physiological and pathophysiological significance. *Immunobiology* **215**(8): 629-646.

Fu J, Astarita G, Gaetani S, Kim J, Cravatt BF, Mackie K*, et al* (2007). Food intake regulates oleoylethanolamide formation and degradation in the proximal small intestine. *The Journal of biological chemistry* **282**(2): 1518-1528.

Gaetani S, Cuomo V, Piomelli D (2003). Anandamide hydrolysis: a new target for anti-anxiety drugs? *Trends in molecular medicine* **9**(11): 474-478.

Giuffrida A, Leweke FM, Gerth CW, Schreiber D, Koethe D, Faulhaber J*, et al* (2004). Cerebrospinal anandamide levels are elevated in acute schizophrenia and are inversely correlated with psychotic symptoms. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **29**(11): 2108-2114.

Gmitrowicz A, Kucharska A (1994). [Developmental disorders in the fourth edition of the American classification: diagnostic and statistical manual of mental disorders (DSM IV -- optional book)]. *Psychiatria polska* **28**(5): 509-521.

Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M*, et al* (2005). Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proceedings of the National Academy of Sciences of the United States of America* **102**(51): 18620-18625.

Gunduz-Cinar O, MacPherson KP, Cinar R, Gamble-George J, Sugden K, Williams B*, et al* (2013). Convergent translational evidence of a role for anandamide in amygdala-mediated fear extinction, threat processing and stress-reactivity. *Molecular psychiatry* **18**(7): 813-823.

Hill MN, Froese LM, Morrish AC, Sun JC, Floresco SB (2006). Alterations in behavioral flexibility by cannabinoid CB1 receptor agonists and antagonists. *Psychopharmacology* **187**(2): 245-259.

Hill MN, Kumar SA, Filipski SB, Iverson M, Stuhr KL, Keith JM*, et al* (2013). Disruption of fatty acid amide hydrolase activity prevents the effects of chronic stress on anxiety and amygdalar microstructure. *Molecular psychiatry* **18**(10): 1125-1135.

Hill MN, McLaughlin RJ, Morrish AC, Viau V, Floresco SB, Hillard CJ*, et al* (2009). Suppression of amygdalar endocannabinoid signaling by stress contributes to activation of the hypothalamic-pituitary-adrenal axis. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **34**(13): 2733-2745.

Hill MN, McLaughlin RJ, Pan B, Fitzgerald ML, Roberts CJ, Lee TT*, et al* (2011). Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**(29): 10506-10515.

Hill MN, Patel S, Campolongo P, Tasker JG, Wotjak CT, Bains JS (2010). Functional interactions between stress and the endocannabinoid system: from synaptic signaling to behavioral output. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**(45): 14980-14986.

Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley D, Mangieri R*, et al* (2005). An endocannabinoid mechanism for stress-induced analgesia. *Nature* **435**(7045): 1108-1112.

Holman EA, Guijarro A, Lim J, Piomelli D (2014). Effects of acute stress on cardiac endocannabinoids, lipogenesis, and inflammation in rats. *Psychosomatic medicine* **76**(1): 20-28.

Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M (2009). Endocannabinoid-mediated control of synaptic transmission. *Physiological reviews* **89**(1): 309-380.

Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A*, et al* (2003). Modulation of anxiety through blockade of anandamide hydrolysis. *Nature medicine* **9**(1): 76-81.

Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the national comorbidity survey replication.(vol 62, pg 593, 2005). *Arch Gen Psychiat* **62**(7): 768-768.

LeDoux JE (2000). Emotion circuits in the brain. *Annual review of neuroscience* **23**: 155-184.

Li H, Penzo MA, Taniguchi H, Kopec CD, Huang ZJ, Li B (2013). Experience-dependent modification of a central amygdala fear circuit. *Nature neuroscience* **16**(3): 332-339.

Lutz B (2009). Endocannabinoid signals in the control of emotion. *Current opinion in pharmacology* **9**(1): 46-52.

Marsicano G, Lutz B (2006). Neuromodulatory functions of the endocannabinoid system. *Journal of endocrinological investigation* **29**(3 Suppl): 27-46.

Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG*, et al* (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature* **418**(6897): 530-534.

Neumeister A, Normandin MD, Pietrzak RH, Piomelli D, Zheng MQ, Gujarro-Anton A*, et al* (2013). Elevated brain cannabinoid CB receptor availability in post-traumatic stress disorder: a positron emission tomography study. *Molecular psychiatry*.

Pan B, Wang W, Long JZ, Sun D, Hillard CJ, Cravatt BF*, et al* (2009). Blockade of 2-arachidonoylglycerol hydrolysis by selective monoacylglycerol lipase inhibitor 4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL184) Enhances retrograde endocannabinoid signaling. *The Journal of pharmacology and experimental therapeutics* **331**(2): 591-597.

Patel S, Hillard CJ (2006). Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling. *The Journal of pharmacology and experimental therapeutics* **318**(1): 304-311.

Patel S, Hillard CJ (2008). Adaptations in endocannabinoid signaling in response to repeated homotypic stress: a novel mechanism for stress habituation. *The European journal of neuroscience* **27**(11): 2821-2829.

Paxinos G, Watson C (1998). *The rat brain in stereotaxic coordinates*, 4th edn. Academic Press: San Diego.

Pecoraro N, Dallman MF, Warne JP, Ginsberg AB, Laugero KD, la Fleur SE*, et al* (2006). From Malthus to motive: how the HPA axis engineers the phenotype, yoking needs to wants. *Progress in neurobiology* **79**(5-6): 247-340.

Piomelli D (2003). The molecular logic of endocannabinoid signalling. *Nature reviews Neuroscience* **4**(11): 873-884.

Piomelli D (2014). More surprises lying ahead. The endocannabinoids keep us guessing. *Neuropharmacology* **76 Pt B**: 228-234.

Pitman RK, Delahanty DL (2005). Conceptually driven pharmacologic approaches to acute trauma. *CNS spectrums* **10**(2): 99-106.

Ramikie TS, Nyilas R, Bluett RJ, Gamble-George JC, Hartley ND, Mackie K*, et al* (2014). Multiple mechanistically distinct modes of endocannabinoid mobilization at central amygdala glutamatergic synapses. *Neuron* **81**(5): 1111-1125.

Ramikie TS, Patel S (2012). Endocannabinoid signaling in the amygdala: anatomy, synaptic signaling, behavior, and adaptations to stress. *Neuroscience* **204**: 38-52.

Sciolino NR, Zhou W, Hohmann AG (2011). Enhancement of endocannabinoid signaling with JZL184, an inhibitor of the 2-arachidonoylglycerol hydrolyzing enzyme monoacylglycerol lipase, produces anxiolytic effects under conditions of high environmental aversiveness in rats. *Pharmacological research : the official journal of the Italian Pharmacological Society* **64**(3): 226-234.

Speckens AE, Ehlers A, Hackmann A, Clark DM (2006). Changes in intrusive memories associated with imaginal reliving in posttraumatic stress disorder. *Journal of anxiety disorders* **20**(3): 328-341.

Steiner MA, Marsicano G, Nestler EJ, Holsboer F, Lutz B, Wotjak CT (2008). Antidepressant-like behavioral effects of impaired cannabinoid receptor type 1 signaling coincide with exaggerated corticosterone secretion in mice. *Psychoneuroendocrinology* **33**(1): 54-67.

Sutt S, Raud S, Areda T, Reimets A, Koks S, Vasar E (2008). Cat odour-induced anxiety--a study of the involvement of the endocannabinoid system. *Psychopharmacology* **198**(4): 509-520.

Zatzick D, Roy-Byrne P (2003). Developing high-quality interventions for posttraumatic stress disorder in the acute care medical setting. *Seminars in clinical neuropsychiatry* **8**(3): 158-167.

Zatzick D, Roy-Byrne PP (2006). From bedside to bench: how the epidemiology of clinical practice can inform the secondary prevention of PTSD. *Psychiatric services* **57**(12): 1726-1730.

Zhang W, Davidson JR (2007). Post-traumatic stress disorder: an evaluation of existing pharmacotherapies and new strategies. *Expert opinion on pharmacotherapy* **8**(12): 1861-1870.

Legends

Figure 1: Rats exposed to TMT show significantly increased anxiety-like behavior and changes in 2-AG mobilization. (a-c) Rats were subjected to EPM 24 h, 7 days or 14 days after exposure to TMT. (a) Ratio of time in open arm, (b) ratio of open arm entry, and (c) anxiety index indicate that exposure to TMT generates long-term anxiety in rats. The anxiety index was calculated as 1-(average of percent time in open arm and percent open arm entry) (n = 8 rats per group). (d) 2-AG content remained elevated in the amygdala for at least 14 days (n = 6 rats per group). (e) Fluctuations in 2-AG level were observed in the hypothalamus for the first 24 h (n = 6 rats per group). In addition to 2-AG content, (f) we observed an increase in c-fos mRNA expression in the amygdala at 24 h but (g) no significant change in CB_1 receptor gene expression. The mRNA data are shown in relative quantity (copies) ratio to GAPDH as normalizer (n = 6-8 rats per group). (h, i) We parsed out TMT-resistant and TMT-sensitive rats.

(h) Anxiety index of TMT-resistant and TMT-sensitive rats indicates that TMT-sensitive rats show heightened level of anxiety-like behavior. (i). TMT-resistant did not exhibit increased levels of 2-AG 7 days after the TMT exposure. N = 4-12 rats per group. Results are expressed as mean±SEM; *P<0.05, **P<0.01.

Figure 2: A single administration of MGL inhibitor, JZL184, exerts anxiolytic-like effects in TMT-exposed rats. (a-c) Acute, single i.p. injection of JZL184 18 h after TMT exposure followed by behavioral test after 6 h after exerts an anxiolytic-like effect. (a) JZL184 (i.p.) administration inhibits brain MGL activity and (b) increase 2-AG content after 6 h (n = 6 rats per group.) (c) Anxiety index data (n = 8-9 rats per group). (d) Administration of JZL184 (i.p.) 18 h after TMT exposure, followed by behavioral tests 7 d post TMT exposure reduces anxiety-like responses in rats (n = 11-12). (e-f) JZL184 (i.p.) administration 6 days before measurement causes no change in brain (e) MGL activity or (f) 2-AG content (n= 6 rats per group). Results are expressed as mean±SEM; *P<0.05, **P<0.01.

Figure 3: The anxiolytic-like effects of JZL184 are CB_1 dependent. (a-c) Intraperitoneal injection of rimonabant 17.5 h after TMT exposure causes no significant behavioral changes. (a) Ratio of time spent in open arm, (b) ratio of open arm entry and (c) anxiety index indicate that rimonabant (1 mg/kg) administration 18 h after TMT exposure has no effect on EPM behavior 7 days later (n= 5-6 rats per group). (d-f) Intraperitoneal injection of rimonabant 30 min prior to a single JZL-184 injection 18 h after TMT exposure blocks the anxiolytic-like effect of JZL-184 that lasts for at least 7 days. (d) Ratio of time spent in open arm, (e) ratio of open arm entry and (f) anxiety index indicate that the anxiolytic-like effects of JZL-184 are CB_1-dependent (n= 11-12 rats per group). Results are expressed as mean ±SEM; *P<0.05, **P<0.01.

Figure 4: Effects of NF1819 on MGL activity and 2-AG mobilization. (a) Intraperitoneal injection of NF1819 (5 mg/kg) lowers rat MGL activity and (b) increases 2-AG content in mouse

brain. (c-e) **I**nfusion of NF1819 into the BLA inhibits local MGL activity and increases 2-AG level. (c) Diagram of the needle tract and location of brain punches taken to measure MGL activity and 2-AG levels in the amygdala. (d) NF1819 infusion into the BLA inhibits MGL activity and (e) increases 2-AG levels at the site of injection (A) but not in nearby tissue (B-D) (n = 4 rats per group). (f-h) Infusion of NF1819 into the third ventricle inhibits MGL activity and increases 2-AG level in the hypothalamus. (f) Diagram of the needle tract and location of brain punches taken to measure MGL activity and 2-AG levels in the hypothalamus. (g) NF1819 infusion into the third ventricle inhibits MGL-activity and (h) increases 2-AG levels in the hypothalamus (n = 4 rats per group). Results are expressed as mean ±SEM; *P<0.05, **P<0.01, ***P<0.001.

Figure 5: The anxiolytic-like effects of MGL inhibition on TMT-induced anxiety-like behavior are mediated by the BLA. (a-c) Infusion of NF1819 into the BLA 18 h after TMT exposure has an anxiolytic-like effect that lasts for at least 7 days. (a) Ratio of time spent in open arm, (b) ratio of open arm entry and (c) anxiety index suggest that the anxiolytic-like effects of the MGL inhibitor occur in the BLA. (d-f) Infusion of NF1819 into the third ventricle has no overt behavioral effect in TMT-exposed rats. (d) Ratio of time spent in open arm, (e) ratio of open arm entry, and (f) Anxiety index (n = 5 per group). Results are expressed as mean ±SEM; n=6-10 rats/group; *P<0.05, **P<0.01.