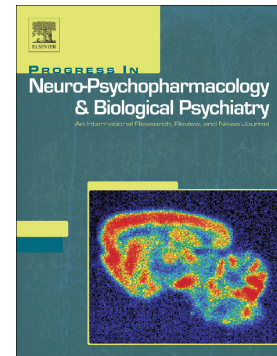


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Zoltan Balogh, Laszlo Szente, Laszlo Biro, Zoltan K. Varga, Jozsef Haller, Mano Aliczki



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Balogh, Zoltan<sup>1,2</sup>; Szente, Laszlo<sup>1,2</sup>; Biro, Laszlo<sup>1,2</sup>; Varga, Zoltan K<sup>1,2</sup>; Haller, Jozsef<sup>3</sup>;  
Aliczki, Mano<sup>1</sup>

<sup>1</sup>Laboratory of Translational Behavioural Neuroscience, Department of Behavioural  
Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences,  
Budapest, Hungary

<sup>2</sup>Janos Szentagothai Doctoral School of Neurosciences, Semmelweis University, Budapest,  
Hungary

<sup>3</sup>Laboratory of Behavioural and Stress Studies, Department of Behavioural Neurobiology,  
Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary

\*Corresponding author: Mano Aliczki, PhD; Institute of Experimental Medicine, Hungarian  
Academy of Sciences, Szigony utca 43; 1083, Budapest, Hungary; aliczki.mano@koki.mta.hu

**Abstract**

Endocannabinoids (eCBs) anandamide (AEA) and 2-arachidonoylglycerol (2-AG) were shown to be involved in the basis of trauma-induced behavioral changes, particularly contextual conditioned fear, however, their ligand-specific effects and possible interactions are poorly understood. Here we assessed specific eCB effects and interactions on acquisition of contextual conditioned fear employing electric footshocks in a rat model. We selectively increased eCB levels by pharmacological blockade of the degrading enzymes of AEA by URB597 and 2-AG by JZL184 before traumatization either systemically or locally in relevant brain areas, the prelimbic cortex (PrL), ventral hippocampus (vHC) and basolateral amygdala (BLA). Following traumatization, a series of contextual reminders were conducted during which conditioned fear was assessed. While systemic URB597-treatment during traumatization only slightly enhanced the acquisition of contextual conditioned fear, administration of the compound in the PrL and vHC led to the acquisition of stable, lasting conditioned fear, resistant to extinction. These effects of URB597 were blocked by simultaneous administration of JZL184. Similar treatment effects did not occur in the BLA. Treatment effects were not secondary to alterations in locomotor activity or nociception. Our findings suggest that AEA and 2-AG functionally interact in the regulation of acquisition of contextual conditioned fear. AEA signaling in the PrL and vHC is a crucial promoter of fear acquisition while 2-AG potentially modulates this effect. The lack of eCB effects in the BLA suggests functional specificity of eCBs at distinct brain sites.

**Keywords:** endocannabinoids; trauma; basolateral amygdala; prelimbic cortex; ventral hippocampus

**Abbreviations:**

2-AG, 2-arachidonoylglycerol; AEA, anandamide; ANOVA, analysis of variance; BLA, basolateral amygdala; CB<sub>1</sub>R, cannabinoid receptor type 1; CeA, central amygdala; dHC, dorsal hippocampus; DMSO, dimethyl sulfoxide; eCB, endocannabinoid; IL, infralimbic i.p., intraperitoneal; mPFC, medial prefrontal cortex; PrL, prelimbic cortex; PTSD, posttraumatic stress disorder; TRPV1, transient receptor potential vanilloid receptor type 1; vHC, ventral hippocampus

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## 1. Introduction

Experiencing a particularly distressing, traumatic event can often lead to the development of the psychiatric condition posttraumatic stress disorder (PTSD). The disorder is characterized by complex, severe symptomatology (APA, 2013), its prevalence is relatively high (Kessler et al., 2005) while its therapy is not sufficiently resolved to this day (Davis et al., 2006).

Therefore, pathomechanism and potential therapeutic targets of PTSD are intensively studied in laboratory animal models.

The endocannabinoid (eCB) system – cannabinoid receptors type-1 (CB<sub>1</sub>R) and 2, eCBs 2-arachidonoylglycerol (2-AG) and anandamide (AEA) and their metabolic enzymes (Devane et al., 1992; Di Marzo et al., 1999; Mechoulam et al., 1995; Munro et al., 1993) – is a major modulator of neuronal plasticity contributing to the regulation of cognitive and emotional processes (Zanettini et al., 2011). Components of the eCB system are abundantly expressed within the neuronal circuits relevant in the behavioral outcome of trauma exposure (Herkenham et al., 1990; Matsuda et al., 1990) and show trauma-induced changes as well (Hauer et al., 2013; Hill et al., 2013; Korem and Akirav, 2014; Marsicano et al., 2002; Morena et al., 2014; Neumeister et al., 2013; Pietrzak et al., 2014) suggesting that eCB signaling is functionally involved in trauma-induced behavioral changes and can be possibly relevant as a therapeutic target in PTSD (Hill et al., 2017).

A number of studies assessed eCB effects in laboratory rodents employing electric footshocks to induce changes resembling the symptomatology of PTSD, most importantly conditioned fear responses to the trauma-associated context (Aliczki and Haller, 2016). While all of these studies concluded that eCB signaling is directly involved in the neurobiological basis of trauma-induced behavioral changes, eCB effects on conditioned fear are still to be clarified in details. Studies employing systemic enhancement or blockade of CB<sub>1</sub>R activity conclusively showed that extinction of conditioned fear is promoted by eCB signaling (Bitencourt et al.,

2008; Chhatwal et al., 2009; Draycott et al., 2014; Kamprath et al., 2006; Laricchiuta et al., 2013; Marsicano et al., 2002; Pamplona et al., 2008; Reich et al., 2008), although similar systemic approaches failed to unambiguously clarify the role of eCB function in acquisition (Marsicano et al., 2002; Pamplona and Takahashi, 2006; Reich et al., 2008; Segev and Akirav, 2011; Sink et al., 2010; Tan et al., 2010) and expression of such responses (Arenos et al., 2006; Draycott et al., 2014; Hofelmann et al., 2013; Jacob et al., 2012; Kamprath et al., 2009; Mikics et al., 2006; Pamplona et al., 2006; Reich et al., 2008). The employment of approaches with higher anatomical or pharmacological specificity more successfully identified the details of eCB involvement in the regulation of conditioned fear.

Employing anatomically localized pharmacological treatments, numerous studies addressed the effects of eCB signaling at distinct brain sites relevant in the basis of conditioned fear, particularly at the prelimbic (PrL) and infralimbic (IL) cortices, dorsal (dHC) and ventral (vHC) regions of the hippocampus and basolateral (BLA) and central (CeA) nuclei of the amygdala. These works suggest that eCB activity at these sites are differentially involved in the dynamics of conditioned fear. ECB activity in the BLA promotes the acquisition of conditioned fear (Tan et al., 2010; Tan et al., 2011). Expression of conditioned fear is dampened by eCB signaling in the IL (Lisboa et al., 2010), dHC (Lin et al., 2011; Segev and Akirav, 2011) and dorsolateral periaqueductal gray (Uliana et al., 2016). Extinction of conditioned fear is promoted by eCB activity in the dHC (de Oliveira Alvares et al., 2008).

Recently, selective pharmacological inhibitors of fatty acid amid hydrolase (FAAH), the degrading enzyme of AEA and monacylglycerol lipase (MAGL), the degrading enzyme of 2-AG became available allowing the assessment of specific effects of AEA and 2-AG, respectively. The most significant effect of such pharmacological compounds in the central nervous system is the elevation of 2-AG (Blankman et al., 2007; Dinh et al., 2002; Makara et al., 2005) and AEA levels (Cravatt et al., 2001; Cravatt et al., 1996; Kathuria et al., 2003;

McKinney and Cravatt, 2005), respectively. While both FAAH and MAGL have several substrates besides AEA or 2-AG, their blockade does not alter the levels of other bioactive lipids, which exert significant, direct effects on any aspects of cognitive or emotional behavior, therefore pharmacological inhibition of FAAH and MAGL is generally accepted to be a valid tool for the assessment of the effects of enhanced AEA and 2-AG signaling, and widely used in the field. Studies employing pharmacological FAAH and MAGL blockade allowing ligand-specific eCB manipulations showed that AEA and 2-AG are differentially involved in the regulation of conditioned fear responses. While specific systemic and intra-IL blockade of FAAH activity attenuated the expression of conditioned fear (Lisboa et al., 2010; Llorente-Berzal et al., 2015), the same process was promoted by systemic MAGL blockade (Llorente-Berzal et al., 2015). Similarly, systemic and intra-dHC FAAH blockade fear extinction (de Oliveira Alvares et al., 2008; Laricchiuta et al., 2013), while it was impaired by MAGL inhibition (Hartley et al., 2016). Ligand-specific eCB effects on the acquisition of conditioned fear are still to be studied. Besides exerting different behavioral effects, recent findings suggest that AEA and 2-AG might also interact in the regulation of behavioral processes as they were shown to directly interact at the cellular level in the hippocampus (Lee et al., 2015).

Taken together, conditioned fear is i.) differentially affected by eCB signaling at distinct brain sites and ii.) the two eCB ligands, which, besides their different effects iii.) potentially interact in the regulation of conditioned fear as well.

We aimed to assess the specific involvement and possible interactions of AEA and 2-AG signaling in the regulation of trauma-induced behavioral changes. As our previous findings showed that acute responses to traumas are modulated by eCB signaling through which it affects long-term trauma-induced changes (Haller et al., 2014) we primarily addressed eCB effects during and shortly following a traumatic event i.e. when acquisition of conditioned

fear occurs. As eCB signaling at different brain sites were shown to differentially affect behavior in numerous cases, studies were conducted in a brain site specific manner. We focused our studies on crucial areas in the acquisition of conditioned fear: i.) the PrL, which site receives sensory information of the conditioned stimuli (e.g. traumatic context) from cortical regions and its excitatory projections activate glutamatergic “fear neurons” in ii.) the BLA, leading to conditioned fear acquisition and expression (Lee et al., 2013). iii.) Besides these areas we studied the vHC as well, which are is reciprocally connected with the PrL and BLA and has a crucial role in context encoding (Huff et al., 2016) in the acquisition of conditioned fear.

## **2. Methods**

### **2.1. Animals**

Subjects were drug and test naïve adult male Wistar rats (Toxi-Coop Zrt.; Hungary) weighing approximately 250 g. Rats were housed individually, laboratory food and water were available *ad libitum*, temperature and relative humidity were kept at  $22 \pm 2$  °C and  $60 \pm 10\%$ , respectively. Rats were housed in normal light cycle with lights on at 08:00 h and lights off at 20:00 h. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine.

### **2.2. Surgical procedures**

Rats were anesthetized with ketamine-xylazine-pipolphen solution (50–10–5 mg/kg; intraperitoneally (i.p.)) and fixed in a stereotaxic frame (David Kopf Instruments). Stainless-steel guide cannulae (21G) were implanted bilaterally, with the cannula tips 2 mm above the PrL (coordinates: anteroposterior, 2.4 mm; mediolateral,  $\pm 1$  mm; dorsoventral, -3.5 mm, from skull surface), vHC (coordinates: anteroposterior, -5.8 mm; mediolateral,  $\pm 5$  mm;



dorsoventral, -7.1 mm, from skull surface) or the BLA (coordinates: anteroposterior, -3 mm; mediolateral,  $\pm$  5.2 mm; dorsoventral, -7 mm, from skull surface) based on the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1998). Reference cannulae tip locations are shown in Figure 3B, 3E and 3H while individual locations are shown in Supplementary figure 1. After surgery, rats were returned to their home cages and subsequently injected intraperitoneally (i.p.) with 0.5 ml of saline and 1 mg/kg Gentamicine in a volume of 1 ml/kg to facilitate clearance of anaesthetics and prevent dehydration and sepsis. Rats were allowed to recover for 7 days following surgery.

### **2.3. Behavioral procedures**

#### *2.3.1. Fear conditioning*

Conditioning was conducted in a separate, dedicated experimental room in bright lighting conditions. Rats were placed in a Plexiglas chamber (30 × 30 × 30 cm), and after 3 min of habituation, three 2.4 mA 2 sec long shocks were administered through the stainless steel grid floor with 30 s inter-shock intervals. Rats remained in the chamber after the last footshock for 60 sec. Such conditions (number, length and intensity) of electric footshocks are well used in similar models (Barsy et al., 2011; Kung et al., 2010; Lin et al., 2011; Mikics et al., 2006; Tulogdi et al., 2012). Non-shocked control rats were placed in the same apparatus for 5 minutes, but shocks were not delivered. The chamber was cleaned with water between sessions. Behavior during fear conditioning was recorded by video camera, and was manually analyzed by an experimenter blinded to experimental conditions with event recorder software (H77, Institute of Experimental Medicine, Hungary). Assessed variables were ambulation (measured in the habituation phase by counting the crossings of projected virtual lines dividing the chamber into 16 equal squares) and duration of freezing (no movements except for breathing).

#### *2.3.2. Contextual reminders*

Contextual reminders were conducted in the same experimental room and lighting conditions as in the case of fear conditioning. Rats were re-exposed to the conditioning chamber with no presentation of footshocks for 5 minutes to measure conditioned fear responses. Behavior during these reminders was recorded by a video camera, and was analyzed by behavior analyzing software EthoVision XT version 11.5 (Noldus Information Technology b.v., Netherlands) for duration of freezing.

### 2.3.3. Nociception measurement in the hot plate test

Changes in nociception were assessed using an increasing-temperature hot plate system (IITC Life Science, Woodland Hills, CA, USA) similarly as described previously by Horvath et al. (Horvath et al., 2014). Rats were placed on the hot-plate apparatus for 3 min of habituation then the plate was heated with a constant rate of 6 °C/min started from 25 °C. Heating was stopped when rats showed nociceptive behavior (i.e. lifting and/or licking the paws), hot plate temperature was recorded as pain threshold then the subject was removed from the apparatus.

## 2.4. Drugs

The MAGL inhibitor JZL184 (PubChem CID: 25021165) (Tocris Bioscience, Bristol, UK) and FAAH inhibitor URB597 (PubChem CID: 1383884) (Sigma Aldrich, Budapest, Hungary) were dissolved in dimethylsulfoxide (DMSO), which was diluted to a final volume with saline that contained 0.4 % methylcellulose. The final solution contained 1.5% DMSO. For systemic administration JZL184, URB597 and vehicle were injected i.p. in a volume of 1 ml/kg body weight 40 min prior to behavioral testing. JZL184 was injected at the dose of 16 mg/kg body weight, URB597 was injected at the dose 0.3 mg/kg body weight. When injected i.p. in these doses in rats, both compounds were shown to markedly elevate the levels of the substrate of their respective target enzyme (*JZL184*: (Lim et al., 2016; Oleson et al., 2012; Seillier et al., 2014; Seillier and Giuffrida, 2018; Wiley et al., 2014); *URB597*: (Seillier and

Giuffrida, 2018)) and alter behavioral processes (*JZL184*: (Lim et al., 2016; Seillier et al., 2014); *URB597*: (Haller et al., 2009; Haller et al., 2013; Kathuria et al., 2003)).

For brain site specific enhancement of eCB signaling *JZL184* and/or *URB597* were infused in a volume of 0.5  $\mu$ l 30 min prior to testing. *JZL184* was injected at the concentration of 1  $\mu$ g/0.5  $\mu$ l; *URB597* was injected at the concentration of 1 ng/0.5  $\mu$ l via a Hamilton microsyringe over 30 sec. In order to prevent backflow and maximize diffusion the injection needle was retained within the cannula for an additional 2 min after drug infusion. Centrally delivered in these doses, both compounds were shown to markedly elevate in the levels of the substrate of their respective target enzyme and alter behavioral processes in rats (Morena et al., 2015). Control animals received vehicle treatments in all experiments throughout our studies.

## 2.5. Experimental design

Procedures were performed during the first 4 hours of the light phase. Animals were randomly assigned to treatment groups with sample sizes 10 to 12. In the case of local drug administration only those rats were included in the final analyses where post mortem histological assessments confirmed correct cannula placement. According to the general procedure employed throughout our studies animals underwent fear conditioning then were re-exposed to the conditioning context daily in the next 7 consecutive days and on the 28th day after conditioning to assess the dynamics of conditioned fear responses. Rats undergoing local pharmacological treatments, were sacrificed after 28th day contextual reminder for assessment of correct cannula localization.

In *experiment 1* FAAH and/or MAGL activity were enhanced 40 min before fear conditioning by systemic administration of *URB597* and/or *JZL184* to study the effects of endocannabinoid signaling on acute and conditioned fear. Animals were randomly assigned to 5 treatment groups: vehicle treated non-shocked controls; vehicle treated shocked controls;

shocked JZL184; shocked URB597; and shocked JZL184 + URB597 treated groups. During all contextual reminders rats were tested drug-free. Detailed design of *experiment 1* is shown in Figure 1A.

In *experiment 2* possible analgesic effects of JZL184- and/or URB597-treatment were assessed using an increasing-temperature hot plate system described above. On the first day, rats received i.p. vehicle treatments then 40 min baseline pain sensitivity was assessed in the hotplate test. On the next day, 40 minutes prior to the hot-plate test JZL184 and/or URB597 were administered i.p. and nociceptive threshold was assessed. Differences between baseline and test day threshold levels indicated treatment-induced changes in pain sensitivity. Detailed design of *experiment 2* is shown in Figure 2A.

In *experiment 3, 4 and 5* we investigated the effects of locally inhibited FAAH and/or MAGL in the PrL, vHC and BLA on acute and conditioned fear. Vehicle, JZL184 and/or URB597 was locally administered 30 min before fear conditioning. Rats were tested drug-free during all contextual reminders. In all other respects *experiment 3, 4 and 5* were identically conducted as described at *experiment 1*. Detailed design of *experiment 3, 4 and 5* is shown in Figure 3A.

## 2.6. Histology

Animals were anesthetized with ketamine–xylazine–pipolphen (50–10–5 mg/kg i.p.) solution and were transcardially perfused with 150 ml ice-cold 0.1 M phosphate-buffered saline followed by approximately 250 ml 4 % paraformaldehyde in 0.1 M phosphate-buffered saline. Brains were removed and immersed in 4% paraformaldehyde (in 0.1 M phosphate-buffered saline). At least 48 h before sectioning, the brains were transferred to a 20% sucrose solution in phosphate-buffered saline at 4 °C for cryoprotection. 30 µm frozen sections were cut in the frontal plane. Section planes were standardized according to the atlas of Paxinos and Watson (1998) and were examined under a light microscope (Olympus BX51). Locations of infusion

needle tips were determined within the PrL, vHC and BLA by an observer blind to experimental conditions. For all experiments, only rats with injection needle tips localized within the targeted region in both hemispheres were included in the data analysis.

## 2.7. Statistical analysis

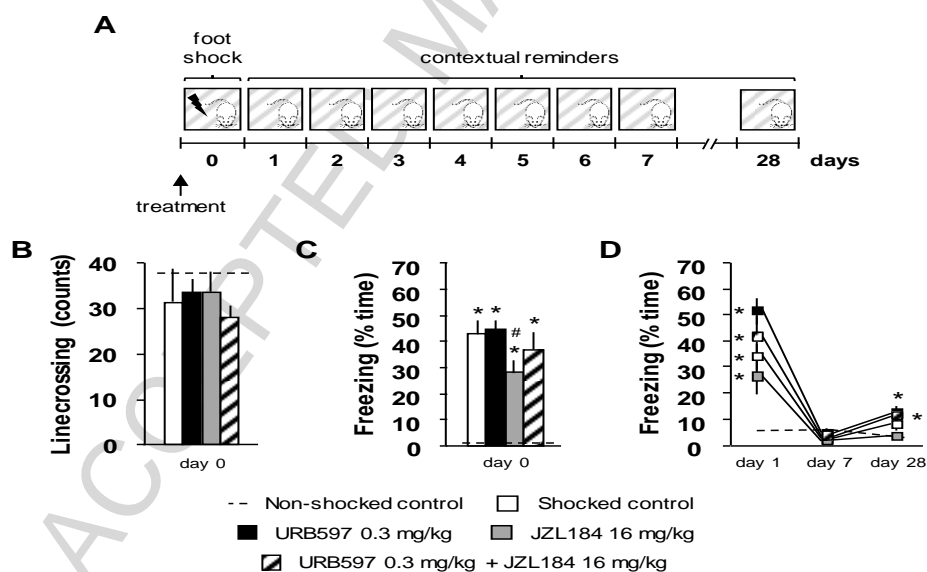
Data were presented as mean  $\pm$  standard error of the mean. Linecrossing counts during the first three minutes of conditioning were evaluated by one-factor analysis of variance (ANOVA) (factor: experimental group), while time spent with freezing during conditioning and throughout the contextual reminders and nociceptive threshold in the hotplate test were analyzed by repeated measures ANOVA (repeated factor: day; non-repeated factor: experimental group). ANOVA assumptions were evaluated by Levene's test, where ANOVA assumptions were not fulfilled, data were square root transformed. Duncan tests were performed for post-hoc analyses when a main effect was significant, and Bonferroni corrections were applied for multiple comparisons. P-values lower than 0.05 were considered statistically significant. All statistical analyses were conducted with Dell Statistica software version 13 (Tulsa, USA).

## 3. Results

### 3.1. The effects of systemic inhibition of FAAH and MAGL on acute fear and acquisition of contextual conditioned fear

Firstly, we studied the effects of systemic pre-conditioning URB597- and/or JZL184-treatment on acute fear responses to traumatic footshocks and the acquisition of contextual conditioned fear. Locomotor activity measured by linecrossing during the first 3 min of the conditioning showed no significant treatment-induced changes ( $F(4,43) = 1.82$ ;  $p = 0.14$ ) (Figure 1B). In contrast, treatment led to significant changes in the duration of freezing behavior during conditioning and contextual reminders ( $F_{\text{treatment}}(4,38) = 10.55$ ;  $p < 0.01$ ;

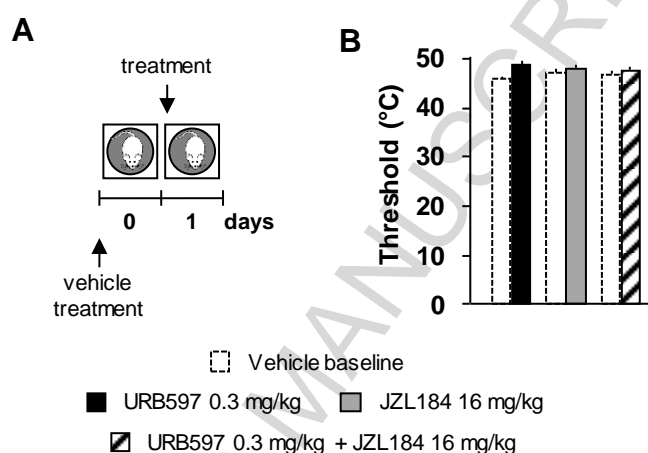
$F_{\text{days}}(8,302) = 64.7178; p < 0.01; F_{\text{group}*\text{days}}(32,304) = 6.91; p < 0.01$ ). Post-hoc comparisons revealed that electric footshocks markedly increased time spent with freezing during conditioning compared to non-shocked controls, which response was dampened by JZL184-treatment (Figure 1C). Duration of freezing behavior during the first contextual reminder was significantly increased by electric footshocks compared to non-shocked controls, which response was unaltered by pharmacological treatments. Freezing returned to non-shocked levels on the seventh day following conditioning in all treatment groups, although extinction took more time in the URB597-treated group throughout the contextual reminders (Supplementary figure 2B). On the 28th day rats receiving pre-conditioning URB597 injections (either alone or concomitantly with JZL184) showed increased freezing levels compared to non-shocked but not to shocked controls (Figure 1D), suggesting a slightly enhanced acquisition of contextual conditioned fear.



**Figure 1. The effects of systemic inhibition of FAAH and MAGL on acute fear and acquisition of contextual conditioned fear.** Experimental design (A); Number of linecrossings during the habituation phase of conditioning (B); Time spent with freezing during conditioning (C); time spent with freezing during contextual reminders on the 1st, 7th and 28th day following conditioning (D). \*, significant difference from non-shocked control; #, significant difference from shocked control treatment group ( $p < 0.05$ ). Sample sizes were 9-11 per treatment group.

### 3.2. The effects of systemic inhibition of FAAH and MAGL on pain sensitivity

Secondly, the effects of systemic URB597- and/or JZL184-treatment on pain sensitivity were studied in the hot plate test. While animals showed a slight decrease in pain sensitivity on the test day compared to baseline ( $F_{\text{days}(1,19)} = 8.38$ ;  $p < 0.01$ ), pharmacological treatments did not alter pain threshold ( $F_{\text{group}(2,19)} = 0.08$ ;  $p = 0.92$ ;  $F_{\text{group*days}(2,19)} = 2.1$ ;  $p = 0.15$ ) (Figure 2B).



**Figure 2. The effects of systemic inhibition of FAAH and MAGL on pain sensitivity.** Experimental design (A); pain threshold in the hot plate test following vehicle control and URB597 and/or JZL184 treatment (B). Sample sizes were 6-8 per treatment group.

### 3.3. The effects of prelimbic inhibition of FAAH and MAGL on acute fear and acquisition of contextual conditioned fear

In our third experiment, we studied the effects of local pre-conditioning URB597- and/or JZL184-treatment in the PrL on acute fear responses to traumatic footshocks and acquisition of contextual conditioned fear. Treatment led to significant changes in the duration of freezing behavior during conditioning and contextual reminders ( $F_{\text{treatment}(4,26)} = 6.44$ ;  $p < 0.01$ ;  $F_{\text{days}(8,208)} = 46.29$ ;  $p < 0.01$ ;  $F_{\text{group*days}(32,208)} = 5.72$ ;  $p < 0.01$ ). Post-hoc comparisons revealed that electric footshocks markedly increased time spent with freezing during

conditioning compared to non-shocked controls, which response was unaltered by pharmacological treatments (Figure 3C). Freezing behavior during the first contextual reminder was significantly elevated in all treatment groups receiving footshocks compared to non-shocked controls, which responses was unaltered by pharmacological treatments. By the seventh day following conditioning, freezing returned to non-shocked control levels in all but the URB597-treated group in which it remained significantly higher than non-shocked and shocked control levels throughout the majority of the contextual reminders (Supplementary figure 3B) suggesting enhanced acquisition of contextual conditioned fear. Concomitant administration of JZL184 blocked this effect of URB597 as freezing in this group did not differ from non-shocked or shocked control levels. Freezing level remained elevated on the 28th day after conditioning in rats receiving pre-conditioning URB597 treatment compared to all treatment groups. Simultaneous pre-conditioning JZL184 injection abolished this effect of URB597 (Figure 3D).

#### **3.4. The effects of ventral hippocampal inhibition of FAAH and MAGL on acute fear and acquisition of contextual conditioned fear**

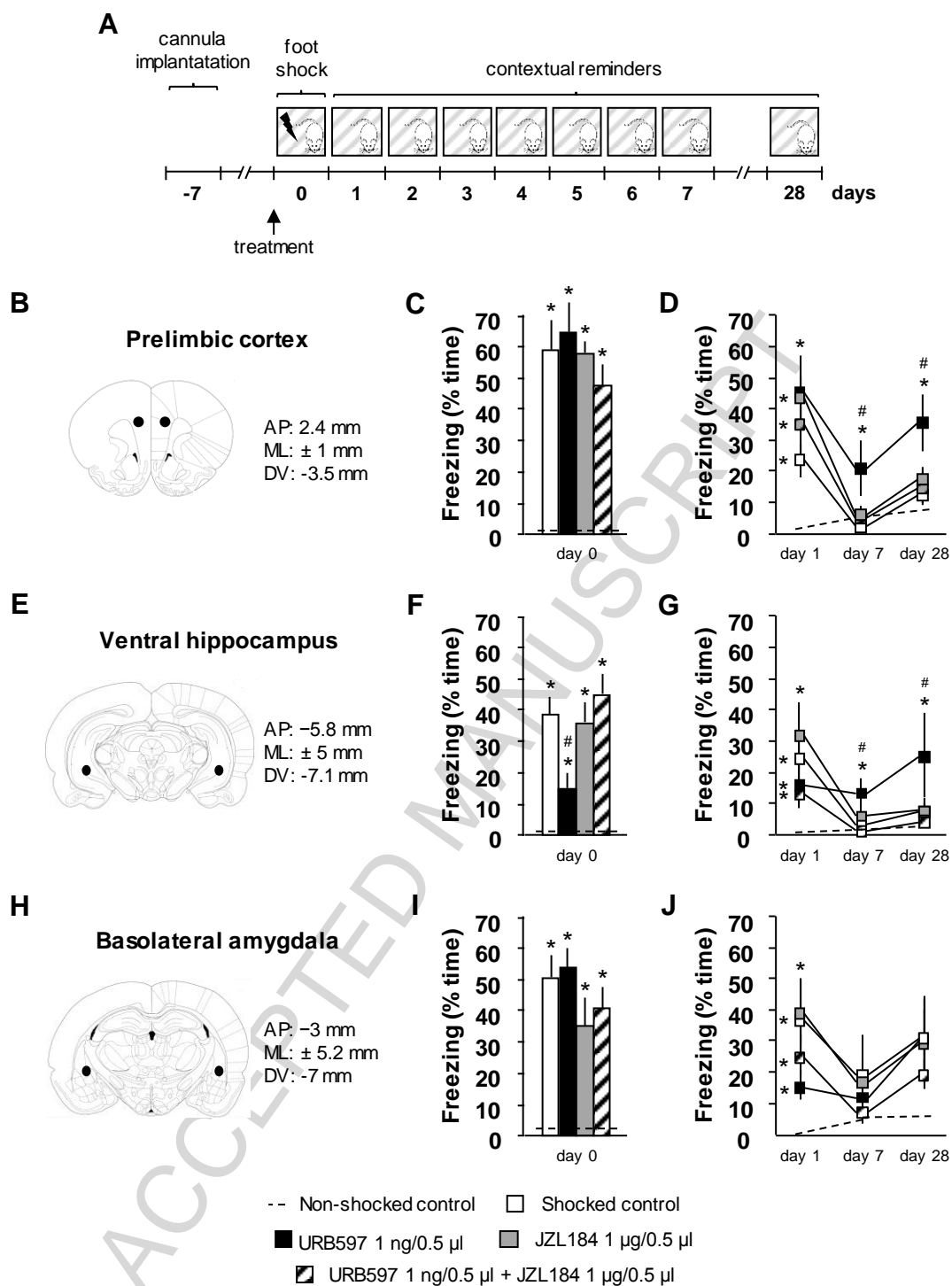
In our next experiment, we studied the effects of local pre-conditioning URB597- and/or JZL184-treatment in the vHC on acute fear responses to traumatic footshocks and acquisition of contextual conditioned fear. Treatment led to significant changes in the duration of freezing behavior during conditioning and contextual reminders ( $F_{\text{treatment}}(4,20) = 5.33; p < 0.01$ ;  $F_{\text{days}}(8,160) = 14.34; p < 0.01$ ;  $F_{\text{group*days}}(32,160) = 4.34; p < 0.01$ ). Post-hoc comparisons revealed that electric footshocks markedly increased time spent with freezing during conditioning compared to non-shocked controls. This response was dampened by URB597-treatment compared to shocked control. Simultaneously administered JZL184 blocked this effect of URB597-treatment without affecting freezing *per se* (Figure 3F). Freezing behavior during the first contextual reminder was significantly elevated in all treatment groups



receiving footshocks compared to non-shocked controls. By the seventh day following conditioning, freezing returned to non-shocked control levels in all but the URB597-treated group in which it remained significantly higher than non-shocked and shocked control levels throughout the contextual reminders (Supplementary figure 3C) suggesting enhanced acquisition of contextual conditioned fear. Concomitant administration of JZL184 blocked this effect of URB597 as freezing in this group did not differ from non-shocked or shocked control levels. Freezing level remained elevated on the 28th day after conditioning in rats receiving pre-conditioning URB597-treatment compared to all treatment groups. Simultaneous pre-conditioning JZL184 injection abolished this effect of URB597 (Figure 3G).

### **3.5. The effects of basolateral amygdalar inhibition of FAAH and MAGL on acute fear and acquisition of contextual conditioned fear**

In our last experiment, we studied the effects of local pre-conditioning URB597- and/or JZL184-treatment in the BLA on acute fear responses to traumatic footshocks and acquisition of contextual conditioned fear. Treatment led to significant changes in the duration of freezing behavior during conditioning and contextual reminders ( $F_{\text{treatment}}(4,22) = 6.23; p < 0.01$ ;  $F_{\text{days}}(8,176) = 9.95; p < 0.01$ ;  $F_{\text{group*days}}(32,176) = 1.73; p = 0.01$ ). Post-hoc comparisons revealed that electric footshocks markedly increased time spent with freezing during conditioning compared to non-shocked control levels which response was unaffected by pharmacological treatments (Figure 3I). Freezing behavior during the first contextual reminder was significantly elevated in all treatment groups receiving footshocks compared to non-shocked controls and returned to non-shocked control levels by the seventh day following conditioning. Freezing during the contextual reminders were unaffected by pharmacological treatments (Supplementary figure 3D). On the 28th day following conditioning no shocked groups showed changes in freezing levels compared to non-shocked controls.



**Figure 3. The effects of locally inhibited FAAH and MAGL on acute fear and acquisition of contextual conditioned fear.** Experimental design (A); reference cannulae locations in the prelimbic cortex (B), ventral hippocampus (E) and basolateral amygdala (H); time spent with freezing during conditioning (pre-conditioning treatment in the prelimbic cortex (C), ventral hippocampus (F), basolateral amygdala (I)); time spent with freezing during contextual reminders on the 1st, 7th and 28th day following conditioning (pre-conditioning

treatment in the prelimbic cortex (**D**), ventral hippocampus (**G**), basolateral amygdala (**J**). AP, anteroposterior from Bregma; ML, mediolateral from midsagittal plane; DV, dorsoventral from skull surface; \*, significant difference from non-shocked control; #, significant difference from shocked control treatment group ( $p < 0.05$ ). Sample sizes were 6-7 per treatment group.

#### 4. Discussion

According to our findings, systemic FAAH blockade during conditioning led to the recovery of contextual conditioned fear suggesting acquisition of a more stable traumatic memory. This effect was even more pronounced when FAAH was locally blocked in the PrL and vHC, in which cases conditioned fear persisted in the studied period, even 28 days after conditioning, suggesting the acquisition of robust, lasting traumatic memory, resistant to extinction. These effects of FAAH blockade in the PrL and vHC were blocked by simultaneous blockade of MAGL. Treatment-effects did not occur in the BLA and were not secondary to alterations in locomotor activity or nociception.

Electric footshocks induced marked acute and conditioned fear responses in our studies consistent with earlier reports (Aliczki and Haller, 2016). Conditioned fear responses disappeared during the seven consecutive contextual reminders and did not return 28 days after conditioning in shocked control subjects. While freezing levels in these animals showed a certain amount of variability across our experiments, the variability was not particularly remarkable and changes of freezing levels across test days showed similar dynamics in all shocked control groups (i.e. significantly higher than non-shocked levels on day 0 and 1, which difference disappeared by day 7 and did not return at day 28).

According to our findings the acquisition of conditioned fear responses were promoted by FAAH blockade. This finding was in line with earlier reports (Mazzola et al., 2009; Wise et al., 2009), especially with studies reporting that such effects are localized in the hippocampus and mPFC (Morena et al., 2014). Interestingly, MAGL blockade in our studies did not alter

aversive memory acquisition *per se*, but blocked the behavioral effects of FAAH blockade in the PrL and vHC. While this findings might seem to contradict earlier reports showing that MAGL blockade promote the consolidation (Ratano et al., 2018) and expression (Llorente-Berzal et al., 2015), while impair the extinction of aversive memories (Hartley et al., 2016), one must note that treatment timing in these studies was immediately after conditioning or before recall, while we temporally limited our treatment effects to conditioning. Interestingly, when pharmacological treatments were delivered before the first contextual reminder in a separate experiment (see Supplementary Material), extinction of conditioned fear was similarly facilitated by FAAH and/or MAGL blockade as well, suggesting that eCBs exert a synergistic, direct effect on this aspect of conditioned fear, furthermore supporting that eCBs differentially affect conditioned fear at different time points.

Surprisingly, pharmacological blockade of FAAH and/or MAGL activity in the BLA did not affect acquisition of conditioned fear, although AEA signaling was specifically shown to promote the formation of aversive memory in this region (Morena et al., 2014). Such contradicting findings regarding the role of eCB signaling in the BLA in the regulation of behavior processes related to aversive contexts were reported earlier. E.g. while intra-BLA AEA infusion was shown to exert anxiolytic effects (Munguba et al., 2011), the involvement of eCBs in the BLA in anxiogenesis was also reported (Di et al., 2016; Morena et al., 2016). While the mechanism through which eCB signaling in the BLA affect traumatic memory acquisition is still to be clarified, as distinct neuronal populations in the BLA are involved in the formation of memories of both positive and negative valence and different aspects of traumatic memory dynamics (Beyeler et al., 2016; Jasnow et al., 2013; Namburi et al., 2016; Namburi et al., 2015). It is possible that treatments non-selectively affected these populations leading to findings contradicting previous reports.

The suggested eCB effects interpreted as direct effects on traumatic memory acquisition cannot be considered secondary to altered perception of footshock-induced pain or changes in motor activity. While acute fear responses were dampened by systemic administration of JZL184 and intra-vHC injection of URB597, we assume that treatment effects on conditioned fear are not the result of such acute effects as an effect dampening fear but leading to the formation of more robust fearful memories is unlikely. This assumption is supported by a previous study from our group in which pre-conditioning URB597-treatment significantly dampened acute fear responses between electric footshocks but conditioned fear responses were unaltered (Haller et al., 2014).

While the molecular details of treatment effects reported here were not directly assessed in the current study, we suggest that blockade of FAAH and/or MAGL, led to behavioral changes *via* increases in eCB levels and therefore enhanced eCB signaling. The most significant effect of our pharmacological approach is the elevation of 2-AG (Blankman et al., 2007; Dinh et al., 2002; Makara et al., 2005) and AEA levels (Cravatt et al., 2001; Cravatt et al., 1996; Kathuria et al., 2003; McKinney and Cravatt, 2005), and while the blockade of both FAAH and MAGL alters the levels of substrates of these enzymes besides AEA or 2-AG, none of these compounds were shown to exert significant, direct effects on any aspects of cognitive or emotional behavior. Regarding the the basis of eCB interactions in the control of conditioned fear acquisition, one possible explanation is that AEA and 2-AG interact *via* the activation of different molecular targets. Such eCB interactions were reported earlier by Lee and colleagues who described that hippocampal tonic 2-AG signaling *via* CB<sub>1</sub>R is antagonized by AEA-induced activation of postsynaptic transient receptor potential vanilloid receptor type 1 (TRPV1) (Lee et al., 2015). As in the same study FAAH blockade-induced enhancement of AEA signaling was showed to attenuate the synaptic effects of tonic 2-AG signaling (i.e. inhibition of GABA release) *via* TRPV1 activation, one might hypothesize that similar eCB

interactions can affect behavioral processes as well. Besides TRPV1, recent data suggest that CB<sub>2</sub>R—although less abundant evidence is available on its expression and function in the brain (Gong et al., 2006; Onaivi, 2006; Onaivi et al., 2006)—may be involved in memory processes as well (Li and Kim, 2016; Stern et al., 2017). A recently emerged study particularly showed that systemic MAGL blockade-induced enhanced 2-AG signaling promoted aversive memory consolidation *via* activation of CB<sub>2</sub>R in rats (Ratano et al., 2018), supporting the theory that CB<sub>2</sub>R function is involved in the modulation of aversive memory. As information regarding the function of this receptor in the central nervous system is still somewhat controversial its involvement in memory processes is needed to be further assessed. Besides exerting their effects through different molecular targets, eCB interactions in the modulation of conditioned fear acquisition can be explained by effects on different neuronal subtypes or subpopulations. CB<sub>1</sub>R activation on different neuronal subtypes were shown to induce different behavioral effects e.g. it is anxiolytic in glutamatergic and anxiogenic in GABAergic neurons (Rey et al., 2012). Similarly, 2-AG was shown to enhance the expression of conditioned fear *via* specifically activating CB<sub>1</sub>R on GABAergic but not glutamatergic neurons (Llorente-Berzal et al., 2015).

## 5. Conclusions

We have shown functional interactions between AEA and 2-AG in the regulation of memory acquisition of a traumatic event. Traumatic memory acquisition is promoted by AEA in the vHC and PrL which effect is dampened by 2-AG. Our findings on eCB interactions suggest an important novel aspect in the eCB control of responses to traumatic events and uncovers a new detail in the basis of trauma-induced behavioral changes which ultimately can contribute to the better understanding the development of PTSD.

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**Author contributions:**

Conceptualization MA, ZB, JH; Methodology MA, ZB; Investigation MA, ZB, LSZ, LB, ZKV; Data Analysis MA; Writing – Original Draft ZB, MA; Writing – Review & Editing MA, ZB, LSZ, LB, ZKV, JH; Visualization MA; Supervision MA; Project Administration MA; Funding Acquisition: MA.

**Conflict of interest:**

The authors declare no conflict of interest.

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**Graphical abstract:**

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**Highlights**

- Endocannabinoids interact in the regulation of conditioned fear acquisition
- Prelimbic and ventral hippocampal anandamide signaling promotes acquisition
- 2-arachidonoylglycerol blocks the effect of anandamide without effects *per se*
- Basolateral amygdalar endocannabinoids do not alter conditioned fear acquisition

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