PROJECT CLOSING REPORT (ZÁRÓJELENTÉS)
for

OTKA project 72621

The role of cannabinoids in the development of trauma-induced behavioral deficits

(Cannabinoidok szerepe a traumák által előidézett viselkedési zavarok kialakulásában)

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2012

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1. SUMMARY

Our studies revealed that neural changes underlying trauma-induced PTSD-like behavioral deficits depend on the nature of the traumatic experience and the challenge subjects are exposed to during behavioral testing. Moreover, neural changes have a temporal evolution that is not reflected at behavioral level. This complexity mirrors the complexity of the human disorder and may explain why the treatment of this disorder is so difficult. Our findings also show that this field needs a complex, multidimensional approach; the frequently used 2-day long conditioned fear test is insufficient to understand the mechanisms of trauma-induced behavioral deficits.

As it regards the role of cannabinoid signaling in trauma-induced behavioral deficits, we suggest that this role can be best described in terms of coping styles. Enhanced anandamide (and possibly 2-AG) signaling increases the predilection of animals to adopt an active coping style. Basic alternative strategies by which individuals respond to environmental challenges (i.e. coping styles) have wide-ranging health implications from immunity to psychopathology, and active coping has been indicated as a therapeutic goal for psychological interventions in various disorders. As such, the enhancement of endocannabinoid signaling may become a therapeutic option in emotional disorders that are characterized changes in coping strategies e.g. in post-traumatic stress disorder.

2. THE MAIN AIMS OF THE PROJECT PROPOSAL

Our project proposal hypothesized that cannabinoid signaling is deeply involved in the development of post-traumatic stress disorder (PTSD), and agents acting on cannabinoid receptors and/or endocannabinoid metabolism constitute a novel approach to the therapy of PTSD. The proposal listed three main aims: (i) to evaluate the effects of cannabinoid-related treatments on various symptoms and models of PTSD, (ii) to understand the brain mechanisms underlying the effects of cannabinoids, and (iii) to compare the efficacy of selected cannabinoid-related treatments with the effects of conventional PTSD therapies. Ultimately our project aimed at understanding the pathomechanisms of PTSD, and at the identification of novel treatment strategies for this disorder.

3. LIST OF STUDIES PERFORMED AND THE MAIN FINDINGS

3.1. Mechanisms underlying behavioral deficits observed in laboratory models of PTSD

3.1.1. The models and methodologies employed

We proposed to study the mechanisms of PTSD-like symptoms by a multidimensional analysis of trauma-related deficits in laboratory models of the disorder. In line with the project proposal, we employed in our studies four laboratory models. In two of these models, traumatic experience consisted of electric shocks. In the remaining two models, traumatic experience was induced by predator exposure and the so-called submersion stress (experience of suffocation). In the case of electric shocks, the behavioral endpoints were social deficits and contextual conditioned fear. Social deficits were studied in two models e.g. the social avoidance and the psychosocial stress paradigms. The conditioned fear test was performed at two time-points, one and 28 days after shocks. In the case of predator exposure and submersion test, the behavioral effects of traumatic experience were studied by means of the elevated plus-maze test of anxiety. In these studies, subjects strongly affected and weakly affected by traumatic experience were studied separately. Trauma-induced changes in addiction-related behaviors were also studied.

Neural mechanisms were studied by c-Fos immunocytochemistry. In certain studies, we identified the activated neurons by means of neuronal markers, while real-time PCR was used as a complementary method in other studies. The local brain administration of compounds was also employed; these studies were presented in chapter 3.2.4.

3.1.2 Findings

3.1.2.1 Social deficits resulting from traumatic experience are mediated by plastic changes in the central and basolateral amygdala

In our study concerning the neural mechanisms of shock-induced social deficits (Mikics et al., 2008), we exposed rats to a single series of electric foot-shocks – a frequently used model of trauma – and studied their behavior in the social avoidance and psychosocial stimulation tests (non-contact versions of the social interaction test) at different time intervals. Social interaction-induced neuronal activation patterns were studied in the prefrontal cortex (orbitofrontal and medial), amygdala (central, medial, and basolateral), dorsal raphe and locus coeruleus. Shock exposure markedly inhibited social behavior in both tests. The effect lasted at least 4 weeks, and amplified over time. As shown by c-Fos immunocytochemistry, social interactions activated all the investigated brain areas. Traumatic experience exacerbated this activation in the central and basolateral amygdala, but not in other regions. The tight correlation between the social deficit and amygdala activation patterns suggest that the two phenomena were associated. A real-time PCR study showed that CRF mRNA expression in the amygdala was temporarily reduced 14, but not 1 and 28 days after shock exposure. In contrast, amygdalar NK1 receptor mRNA expression increased throughout. Thus, the trauma-induced social deficits appear to be associated with, and possibly caused by, plastic changes in fear-related amygdala subdivisions.

3.1.2.2 Anxiety-like consequences of predator exposure are mediated by the medial amygdala and prefrontal cortex, whereas the behavioral consequences of submersion stress depend on the serotonergic cells of the dorsal raphe

Our findings using predator exposure and submersion stress as traumatic events were summarized in two publications (Adamec et al., 2012a, 2012b). This study was performed in cooperation with the group of Robert Adamec, Canada. The effects of the two stressors were studied in comparison. This first study (Adamec et al., 2012a) had two purposes. First: to compare predator and water submersion stress cFos activation in medial prefrontal cortices (mPFC) and the medial amygdala (MeA). Second: to identify markers of vulnerability to stressors within these areas. Rats were either predator or submersion stressed and tested 1.75 h later for anxiety. Immediately thereafter, rats were sacrificed and cFos expression was examined. Predator and submersion stress equally increased anxiety-like behavior in the elevated plus maze (EPM) and hole board. To examine vulnerability, rats which were less anxious (LA) and more (highly) anxious (MA) in the EPM were selected from among handled control and stressed animals. LA stressed rats were considered stress non-responsive while MA stressed rats were considered stress responsive. Predator stress, but not submersion stress, activated MeA cFos. CFos expression of mPFC cells was elevated in LA rats and reduced in MA rats in predator stressed animals only, correlating negatively with anxiety. These findings are consistent with data implicating greater mPFC excitability in protection against the effects on affect of traumatic stress. The findings also suggest that this conclusion is stressor specific, applying to predator stress but not submersion stress. Both stressors have been suggested to model hyperarousal and comorbid anxiety aspects of PTSD in humans. Hence the use of these paradigms to identify brain bases of vulnerability and resilience to traumatic stress in PTSD has translation potential. On the other hand, our evidence of stressor specificity of vulnerability/resilience markers raises a caution. The data suggest that preclinical markers of vulnerability/resilience in a given stress paradigm are at best suggestive, and translational value must ultimately be confirmed in humans. The second study of the same series (Adamec et al., 2012b) was performed under very similar conditions, but here we focused on the brainstem, namely on the dorsal raphe where serotonergic and non-serotonergic neurons were differentially studied and the locus coeruleus. LA and MA rats did not differ in cFos expression in any brain area, though stressors did increase cFos cell counts in all areas over

controls. Intriguingly, the number of serotonergic DR neurons not activated by stress predicted degree of anxiety response to submersion stress only. LA submersion stressed rats had more serotonergic cells than all other groups, and MA submersion stressed rats had fewer serotonergic cells than all other groups, which did not differ. Moreover, these cell counts correlated with EPM anxiety. We conclude that a surplus of such cells protects against anxiogenic effects of submersion, while a paucity of such cells enhances vulnerability to submersion stress. Other data suggest serotonergic cells may exert their effects via inhibition of dorsolateral PAG cells during submersion stress. Findings are discussed with respect to serotonergic transmission in vulnerability to predator stress and relevance of findings for post traumatic stress disorder (PTSD). This article was part of a Special Issue of Neuropharmacology entitled 'Post-Traumatic Stress Disorder'.

3.1.2.3. The control of shock-induced conditioned fear undergoes rapid changes after shock exposure; lasting conditioned fear implicates the medial prefrontal cortex, medial and basolateral amygdala, anterior hypothalamic nucleus, median raphe and periaqueductal gray

Our last study in this series (Tulogdi et al., 2012) was prompted by discrepant earlier findings concerning the role of various brain regions in conditioned fear. We hypothesized that discrepant findings were due to dynamic neural changes that followed shocks, and a more consistent picture would emerge if consequences were studied after a longer interval. Therefore, we exposed rats to a single session of footshocks and studied their behavioral and neural responses one and 28 days later. The neuronal activation marker c-Fos was studied in 24 brain regions relevant for conditioned fear, e.g. in subdivisions of the prefrontal cortex, hippocampus, amygdala, hypothalamic defensive system, brainstem monoaminergic nuclei and periaqueductal gray. The intensity of conditioned fear (as shown by the duration of contextual freezing) was similar at the two time-points, but the associated neuronal changes were qualitatively different. Surprisingly, however, Multiple Regression Analyses suggested that conditioned fear-induced changes in neuronal activation patterns predicted the duration of freezing with high accuracy at both time points. We suggest that exposure to electric shocks is followed by a period of plasticity where the mechanisms that sustain conditioned fear undergo qualitative changes. Neuronal changes observed 28 days but not 1 day after shocks were consistent with those observed in human studies performed in PTSD patients.

3.1.2.4. Traumatic experience induced by electric shocks durably affect behavior in tests relevant to drug abuse in conjunction with the development of post-traumatic stress disorder-like behavioral dysfunctions

In addition to the studies listed above, we also studied the impact of traumatic experience on addiction-related behaviors. Rats exposed to 10 shocks of 3 mA over 5 min showed a robust conditioned fear 28 days later, which confirms the traumatic nature of shock exposure. A different set of rats was studied in the conditioned place preference paradigm beginning with the 27th post-shock day. 10mg/kg morphine induced a marked place preference in both shocked and non-shocked rats. Although the magnitude of place preference was not affected, extinction was markedly delayed in shocked rats. We also investigated tolerance to the hyperthermic effects of morphine. A low dose (5mg/kg) that was administered 4 weeks after shock exposure robustly increased body temperature in both shocked and non-shocked rats. Repeated injections resulted in a mild tolerance in non-shocked controls; yet, morphine readily increased body temperature in these rats on the 5th day of injections. In contrast, the temperature-heightening effect of morphine was abolished in shocked rats after 2 days. Thus, shock exposure considerably delayed the extinction of place preference induced by, and dramatically accelerated the tolerance to the effects of, morphine. Our study shows that electric shocks durably affect behavior in tests relevant to drug abuse in conjunction with the development of post-traumatic stress disorder-like behavioral dysfunctions. This study aimed at describing the phenomenon, the neural background of which was planned to be studied

later. We discovered in the meantime that endocannabinoid signaling affects coping responses, which has a large impact on PTSD-like behavioral dysfunctions, for which our attention was shifted to this issue and the "neurochemical mechanism part" of this particular study was not performed. We mention that such studies were not planned.

3.1.3 Conclusions

We studied here the neuronal background of various trauma-induced behavioral deficits in four laboratory models of PTSD. Surprisingly, large differences were observed between the four models; moreover, the neuronal background of behavioral deficits of different nature (e.g. social fear vs. conditioned fear) was different even when the traumatic experience was similar. We also revealed that neural mechanisms undergo rapid changes after the traumatic experience; the very same behavioral response (e.g. freezing in shock-associated environments) is controlled by different mechanisms 1 and 28 days after shocks.

The complexity of trauma-induced neural changes mirrors the complexity of the human disorder and may provide a hint on the reasons why the treatment of this disorder is so difficult. At the same time our findings demonstrate that this field needs a complex, multidimensional approach; the frequently used 2-day long conditioned fear test is insufficient to understand the mechanisms underlying trauma-induced behavioral deficits.

3.2.Cannabinoid mechanisms underlying anxiety and PTSD-like behavioral dysfunctions in laboratory models

3.2.1. Models and methodologies employed

The main PTSD model employed in these studies was the conditioned fear paradigm. Consequences were studied either one or several weeks (usually 4) after electric shocks. To study the involvement of endocannabinoids we used CB1-KO mice, and various cannabinoid ligands. The main pharmacological tool employed in these studies was, however, the FAAH inhibitor URB597, which indirectly enhances endocannabinoid –particularly anandamide– signaling. Compounds were administered either systemically or locally into distinct brain regions. The behavioral tests used in these studies include the elevated plus-maze test of anxiety, the conditioned fear paradigm a laboratory model of PTSD, the forced swimming test of depression, and two tests of coping. Beyond behavioral methods, we also employed hormone measurements, *in vivo* biotelemetry, and electrophysiological studies to evaluate the short and long-term consequences of traumatic experiences.

3.2.2. Endocannabinoid signaling and 5-HT_3 receptor-mediated serotonergic neurotransmission interactively control anxiety and the long-term effects of traumatic experience

The first series of studies focused on the interaction between cannabinoid signaling, serotonergic neurotransmission mediated by 5-HT3 receptors, and GABAergic mechanisms underlying anxiety and PTSD-like behavioral dysfunctions in mice and rats (Mikics et al., 2009). Neuroanatomic findings revealed that CB1 cannabinoid and 5-HT3 receptors are co-expressed by a subtype of gamma-amino butyric acid (GABA)ergic interneurons in the prefrontal cortex, hippocampus, and basolateral amygdala, three brain regions that are crucial for the control of anxiety. In these regions, serotonergic inputs increase GABA release through 5-HT3 receptors, the phenomenon being retrogradely controlled by cannabinoid neurotransmission. This suggests a functional interaction between 5-HT3 neurotransmission and CB1 signaling. In a first attempt to investigate the behavioral relevance of these interactions, we studied the effects of the selective 5-HT3 agonist 1-(m-chlorophenyl)-biguanide (mCPBG), on plus-maze behavior in NMRI, CD1 wild type, and CB1-KO mice. The genetic disruption of CB1 receptors consistently increased anxiety. This effect was significantly decreased by the 5-HT3 agonist, mCPBG. The dose-response curve was bell-shaped. Surprisingly, mCPBG did not affect the behavior of CD1 wild type and NMRI mice.

We hypothesize that in the aforementioned regions, 5-HT3 activation decreases anxiety by promoting GABA release, but this effect is dampened by CB1 signaling. The disruption of CB1 receptors in CB1-KOs released GABA neurons from retrograde inhibition and made the effects of 5-HT3 stimulation conspicuous. Altogether, our findings revealed a functional interaction between 5-HT3 neurotransmission and CB1 signaling. Besides this interaction being an interesting aspect of anxiety control, it may also explain the notoriously inconsistent effects of 5-HT3 ligands on anxiety. If 5-HT3 neurotransmission and CB1 signaling interact, the anxiety-related effects of 5-HT3 ligands may depend on species, strain, and situation-related differences in both 5-HT3 and CB1 receptor expression and function.

The same interaction was studied later in the conditioned fear paradigm, a putative laboratory model of PTSD (Mikics and Haller in preparation; estimated publication year: 2013). Although the findings of this study are not fully available yet, the study clearly showed that similar to anxiety PTSD-like behavioral dysfunctions strongly depended on the interaction between endocannabinoid signaling and serotonergic mechanisms mediated by 5-HT_3 receptors.

3.2.3. The indirect modulation of endocannabinoid signaling by the inhibition of the anandamide-degrading enzyme FAAH - complex and context-dependent effects on anxiety

In an attempt to characterize more specifically the role of endocannabinoid system in anxiety and PTSD-like behavioral dysfunctions, we employed the FAAH inhibitor URB597 in our studies (Scherma et al., 2008b). When the anxiety-related effects of the endocannabinoid anandamide and the FAAH inhibitor URB597 were evaluated in a light/dark box, both a low anandamide dose (0.3 mg/kg) and URB597 (0.1 and 0.3 mg/kg) produced anxiolytic effects when given alone, but produced anxiogenic effects when combined. A higher dose of anandamide (3 mg/kg) produced anxiogenic effects and depressed locomotor activity when given alone and these effects were potentiated after URB597 treatment.

In another study, we demonstrated that URB597 affects anxiety in a highly context-dependent manner (Haller et al., 2009). We found that URB597 (0.1-0.3 mg/kg) did not produce anxiolytic effects when the aversiveness of testing procedures was minimized by handling rats daily before experimentation, by habituating them to the experimental room, or by employing low illumination during testing. In contrast, URB597 had robust anxiolytic effects when the aversiveness of the testing environment was increased by eliminating habituation to the experimental room or by employing bright lighting conditions. Unlike URB597, the benzodiazepine chlordiazepoxide (5 mg/kg) had anxiolytic effects under all testing conditions. The anxiolytic effects of URB597 were abolished by the cannabinoid CB1-receptor antagonist AM251, showing that they were mediated by CB1 receptors. Close inspection of experimental conditions employed in earlier reports suggests that conflicting earlier findings with URB597 can be explained by different testing conditions, such as those manipulated in the present study. Our findings showed that FAAH inhibition does not affect anxiety under mildly stressful circumstances but protects against the anxiogenic effects of aversive stimuli. This series of studies led us to conclude that endocannabinoid signaling affects in fact coping strategies and affects the development of PTSD-like behavioral dysfunctions *via* this route. Our findings related to this phenomenon will be presented below.

3.2.4. The interaction between endocannabinoid signaling, coping with aversive stimuli, and long-term deficits induced by traumatic experience

We will present the findings of this last experimental series of the project in detail, partly because the findings were not yet published, and partly because the findings of these studies represent the quintessence of the whole project, and summarize our most important finding, namely the impact of cannabinoids on coping styles and the relevance of this phenomenon for post-traumatic stress disorder.

3.2.4.1 Theoretical premises

Active and passive coping are two distinct phenotypes, which differ in the way in which challenges are dealt with (Koolhaas et al., 1999; Koolhaas, 2008). In novel situations, active copers base their behavior on routines that are weakly influenced by environmental stimuli and attempt to control challenges when they occur. Thus, behavior is internally driven and problem-oriented in active copers. In contrast, passive copers are governed by environmental stimuli and tend to answer challenges by inactivity, demonstrating externally-driven and avoidant behavior. These temporally stable behavioral phenotypes were shown to have adaptive significance in animals, while in humans (where they are often depicted as Type "A" and Type "C" coping) they influence disease susceptibility and resilience under adverse conditions(Irvine et al., 1982; Kessler et al., 1985; Koolhaas et al., 1999; Koolhaas, 2008; Stewart and Yuen, 2011; Temoshok, 2000); moreover, coping styles are believed to predict disease-induced decreases in quality of life more accurately than disease severity (Pucheu et al., 2004; Westerhuis et al., 2011). Consequently, interventions promoting active coping styles –which are associated more favorably with resilience–, were proposed as therapeutic goals in a variety of severe physical and mental diseases (Cooke et al., 2007; Sawyer et al., 2009; Tiemensma et al., 2011; Westerhuis et al., 2011). Disparate findings suggest that coping styles are determined at the level of the prefrontal cortex (Stalnaker et al., 2009) and are influenced by serotonergic neurotransmission (Wilhelm et al., 2007), but their neural mechanisms remain poorly understood. Endocannabinoids appeared to be good candidates for controlling coping responses as these signaling molecules are synthesized on demand in response to aversive environmental stimuli (Hohmann et al., 2005; Kirkham et al., 2002; Marsicano et al., 2002; Walker et al., 1999) and serve as feedback mechanisms that reduce challenge-induced neuronal excitations (Hohmann et al., 2005; Gerdeman and Lovinger, 2001; Straiker and Mackie, 2009). We have recently shown within this project that anandamide decreases anxiety-like behavior indirectly by blunting the behavioral impact of aversive stimuli (Haller et al., 2009), and the same may be true for the other major endocannabinoid 2-arachidonoylglycerol (Sciolino et al., 2011). This suggested that endocannabinoids determine coping styles by influencing the impact of the environment on behavior. We performed a series of studies to investigate this hypothesis and to establish its relevance for conditioned fear a putative model of post-traumatic stress disorder.

3.2.4.2 Findings

<u>FAAH inhibition promotes independence from anxiety-linked stimuli in rats</u>

We first tested the effects of URB597 in the elevated plus-maze model of anxiety under three sets of conditions. At one extreme, we studied rats under non-aversive conditions: low light (<5 lx) in a habituated testing environment. At the other extreme, we studied rats under aversive conditions: high light (>300 lx) in an unfamiliar environment. The third condition involved an intermediate level of aversiveness: a habituated environment but with high lighting. These experimental conditions elicit differential HPA-axis activation consistent with the intended level of aversiveness (Haller et al., 2009). In line with earlier observations, URB did not affect anxiety under the intermediate, mildly aversive condition (habituated environment, high light) but decreased anxiety under the aversive condition (Haller et al., 2009) (Figs 1 a-c)[1]. But, compared to vehicle-treated rats, URB597 induced anxiety-like behavior when given under the least aversive conditions (habituation, low light). This surprising finding could lead to the conclusion that anandamide can have either anxiogenic or anxiolytic effects depending on environmental conditions. However, an alternative interpretation is suggested by the fact that the behavior of the URB597-treated rats was about the same under all three conditions, while the vehicle-treated rats showed anxiety-like effects

[1] Figures were presented at the end of this report

in the intermediate and aversive conditions (Figs 1d-f; open arm exploration dramatically decreased in parallel with the increase in the aversiveness). Thus, vehicle treated rats reacted strongly to the environment, but this reactivity was reduced and abolished in rats treated with 0.1 and 0.3 mg/kg URB597, respectively.

To elucidate the brain areas involved in this novel behavioral response, we delivered URB597 into areas known to be involved in anxiety, the medial prefrontal cortex and the amygdala. The prefrontal cortex was also implicated in defining coping styles (Stalnaker et al., 2009). The drug was delivered through chronic implants due to the aversiveness (handling, restraint) associated with acute local injections, which would have interfered with the testing conditions that were keys to these studies. When delivered into the medial prefrontal cortex, URB597 produced a profile of behavioral effects very similar to that seen after acute intraperitoneal injections (Figs 2a-c). In contrast, URB597 did not alter the responsiveness of rats to environmental conditions when delivered into the amygdala (Figs 2d-f). Taken together, these findings suggest that URB597 decreased reactivity to environmental conditions by a mechanism involving the medial prefrontal cortex (involved in both anxiety and coping) but not the amygdala (involved in anxiety mainly). URB597 was clearly detectable at the end of the experiment in the prefrontal cortex where anandamide showed the expected changes (Fig. 2g).

Anandamide-induced control over challenging situations in rats

We studied the effects of UB597 in the tail-pinch test, where a small clamp is attached to rats' tails and coping styles are indicated by activity directed at removing the clamp (Giorgi et al., 2003). This paradigm was chosen over more widely used tests of coping because it allows repeated testing to detect changes in coping styles within subjects. Coping style in the tail-pinch test was characterized by the ratio between clamp-oriented (e.g. gnawing the clamp) and environment-oriented (e.g. exploration) behaviors. When rats were repeatedly treated with vehicle, the behavioral profile of individuals was highly consistent over time, and the ratio clearly differentiated three types of individuals in terms of coping style: active (more than 60% of time spent with clamp gnawing; ratio >1.5), passive (more than 60% of time spent with exploration; ratio <0.66) and mixed style (0.66<ratio>1.5).

In contrast to vehicle treatments, URB597 dose-dependently altered coping styles, such that behavioral differences between active, passive and mixed copers disappeared (Fig. 3a). Generally, URB597 shifted the passive and mixed copers towards a more active style, while the active copers remained active or shifted slightly toward a less active style. The cannabinoid CB1 receptor antagonist/inverse agonist rimonabant (at a dose that had no effect when given alone) abolished the effects of URB597 on coping (Fig. 3b). The frequency distribution of coping styles in the above two studies revealed a significant shift towards active coping styles after URB597 treatment (Fig. 3c), a behavioral change not secondary to altered sensitivity to clamp-induced discomfort, as neither coping style nor URB597 affected pain perception (Fig. 3d). Since the experimental situation used to assess coping was highly aversive and therefore conducive to URB597 having an anxiolytic effect, we also studied the effects of the anxiolytic benzodiazepine chlordiazepoxide; the results of this experiment led to the conclusion that alterations in coping styles are not a general consequence of anxiolytic treatments (Fig. 3e). Taken together, these findings point towards a strong involvement of anandamide signaling in shaping coping styles by a mechanism involving the CB1 receptor. Enhanced anandamide signaling by FAAH inhibition in rats eliminates differences in coping styles by promoting active coping overall.

FAAH inhibition and coping in mice

The behavioral effects of cannabinoids may depend on the relative cannabinoid responsiveness of GABAergic and glutamatergic neurotransmission, and marked species differences have been reported in this respect (Haller et al., 2007). Therefore, we employed

mice to investigate whether the findings obtained in rats are valid in this species. We also changed the experimental paradigm to check whether the impact of URB597 on stimulus responding is dependent on the sensory modality. Mice were studied in the forced swimming test of depression-like behavior under three different water temperatures. In this species, floating —a depression-like behavior in this test— is markedly decreased when water temperature is increased from the usual 20-25^{o}C to about 35^{o}C (Arai et al., 2000). We also found that mice floated significantly less in warm (35^{o}C) than in cold water (15 or 25^{o}C) (Fig 4a). In addition, the corticosterone response to forced swimming was reduced, showing that mice felt more comfortable in warm as compared to cold water (Fig 4b). FAAH inhibition by URB597 did not affect behavior when mice swam at low temperature, but it increased floating and decreased struggling and swimming at 35^{o}C (Figs 4c-e). It occurs, however, that this depression-like effect —surprising for a putative antidepressant— was secondary to decreased responsiveness to water temperature. While vehicle treated mice strongly responded to it, URB597-treated mice failed to do so (Figs 4f-h). These findings suggest that URB597 abolishes the behavioral consequences of comfortably warm water which mirrors the findings obtained in rats exposed to the elevated plus-maze under comfortable conditions.

Encouraged by the parallelisms between rat plus-maze and mouse forced swimming findings, we investigated the impact of URB597 on coping in mice. We employed the back-test that was initially developed for piglets (Ruis et al., 2001), and was later adapted to rats (Hawley et al., 2010) to evaluate coping styles by assessing behavioral resistance to a forced unnatural position. After submitting mice repeatedly to this test, we concluded that their behavior is consistent over time, and that coping styles are clearly separable and remain constant after repeated testing (Figs 5a, b). Thus, this test –like the tail-pinch test– offered the advantage of following alterations in coping in the very same animals. In a second experiment, coping styles again remained constant when tests were preceded by vehicle treatment (Fig 5c). In contrast, FAAH inhibition by URB597 promoted active coping in mice that showed passive coping during the vehicle trial (Fig 5d).

The emotional impact of shock reminders - the role of endocannabinoid signaling

It has been postulated that behavioral/emotional vulnerability to excessively strong (traumatic) stressors are strongly modulated by coping styles. Particularly, coping with a severe stressor has an impact on both the development of post-traumatic disorder (PTSD) in humans (Yehuda et al., 2006; Gil and Caspi, 2006) and the development of PTSD-like behavioral dysfunctions in laboratory models (Walker et al., 2008). Prompted by these earlier findings, we hypothesized that FAAH inhibition would ameliorate the behavioral and emotional impact of traumatic stressors by interacting with coping styles. Therefore, we investigated the effects of URB597 in the shock-induced contextual conditioned fear paradigm, a model frequently used to model PTSD and to study its neurobehavioral mechanisms in the laboratory (Ciocchi et al., 2010). Treatment with URB597 was given only once, 40 min before the conditioning phase (i.e. before shocks). Each shock elicited runs of about 50 cm and occasional escape jumps that were not affected by URB597 treatment (Fig 6a). This shows that in line with the data in rats (Fig 3d) pain sensitivity was not affected by this compound. However, FAAH inhibition significantly affected the behavior observed between shock presentations. That is, locomotion during the 30-s breaks that separated shocks was gradually reduced from about 10 cm to almost nil in vehicle-treated mice, but there were two waves of locomotion bouts in URB597-treated mice, resulting in an overall increase in locomotion (Fig 6b). Interestingly, the direction of exploration was also changed. While vehicle-treated mice directed their exploratory activity mostly in the air, URB597-treated mice spent an equal amount of time exploring the space between and beneath the metallic grid by which shocks were delivered (Fig 6c). Freezing was also reduced by FAAH inhibition (Fig 5d). Overall, our findings show that the behavioral inhibition induced by shocks was

considerably ameliorated by URB597. Thus, FAAH inhibition resulted in a more active way of coping with this demanding situation.

Mice that were exposed to shocks under the influence of URB597 showed slightly but significantly reduced freezing when re-exposed to the shocking cage two weeks later (Fig 6d). Notably, mice received no treatment at this time-point. Sleep was investigated by electroencephalographic analysis to assess the emotional impact of shock and contextual reminders as it was shown that both inescapable shocks and contextual reminders increase wakefulness and produce fragmented sleep (Philbert et al., 2011; Sanford et al., 2003). Surprisingly, the impact of shocks on sleep was not particularly strong: during the 30 min that followed shock exposure, 20-30% of the time was already spent sleeping, and pre-shock levels of 80% were resumed within about 90 min (Fig 6e). In contrast, sleep was almost completely abolished during the 30 min that followed the contextual reminder, despite the fact that no shocks were delivered this time. At the end of the investigated period, control mice slept significantly more after the reminder than after the shock, a possible rebound effect. No similar changes were noticed in the group treated with URB597, where wakefulness elicited by the shock and by the reminder were similar (Fig. 6e). Shock exposure also resulted in fragmented sleep that lasted about 120 min as shown by the number of awakenings, which increased despite a substantial decrease in sleep duration (Fig. 6f). The contextual reminder did not change this pattern in mice treated with vehicle before shock presentation (i.e. 14 days earlier). In contrast, mice submitted to shocks concurrently with FAAH inhibition showed robust sleep fragmentation after shocks but not after the contextual reminder, demonstrating a marked decrease in the emotional impact of the reminder. Taken together, these findings show that FAAH inhibition during shock exposure lessened the immediate behavioral suppressive effects of electric shocks, and this single treatment also ameliorated the long-term behavioral and emotional disturbances resulting from shock exposure.

3.2.4.3 Conclusions

Endocannabinoids affect the function of many neurotransmitter systems, and these systems sometimes play opposing roles. This inherently leads to complex and situation-dependent effects (Fride et al., 2005; Zanettini et al., 2011). Accumulating evidence suggests that the role of cannabinoid signaling may be general rather than linked to specific behaviors. For example, it has recently been proposed that cannabinoid signaling promotes emotional homeostasis (Marco and Viveros, 2009) and works as an emotional buffer system that ensures an appropriate reaction to stressful events (Ruehle et al., 2011). The findings resulting from this project suggest that these general roles can be best described in terms of coping styles, which explains contrasting findings and unifies earlier concepts. Enhanced anandamide (and possibly 2-AG (Sciolino et al., 2011) signaling promotes both intrinsically driven behaviors that buffer the impact of environmental stimuli and attempts to control challenging situations (i.e. it increases the predilection of animals to adopt an active coping style). Moreover, one can bridge the basic mechanism of action of cannabinoids (the blunting of excessive neuronal activation (Wilson and Nicoll, 2001) and the preferential location of CB1 receptors in the limbic system (Herkenham et al., 1990), and hypothesize that the main role of cannabinoid signaling is to blunt environmentally-induced neuronal activations, and consequently to increase the relative weight of intrinsic controlling mechanisms and produce a more active coping style. This hypothesis may shed new light on the clinical prospects of agents that indirectly increase endocannabinoid signaling. Basic alternative strategies by which individuals respond to environmental challenges (i.e. coping styles) have wide-ranging health implications from immunity to psychopathology(Irvine et al., 1982; Kessler et al., 1985; Koolhaas et al., 1999; Koolhaas, 2008; Stewart and Yuen, 2011; Temoshok, 2000). In fact, promoting active coping has been indicated as a therapeutic goal for psychological interventions in various disorders (Cooke et al., 2007; Sawyer et al., 2009; Tiemensma et al.,

2011; Westerhuis et al., 2011). As such, the enhancement of endocannabinoid signaling may become a therapeutic option in emotional disorders that are characterized by excessive responses to environmental signals.

3.3. Other findings and results of the project

While performing the undertaken tasks, we carried out a series of experiments that were aimed at preparing certain phases or tasks of the project. Although the findings of these experiments were not always useful from the point of view of the main aims, they still provided interesting findings and as such were published. Importantly, several of these studies became starting points of new projects.

3.3.1. The NR2B NMDA receptor subunit as a novel treatment approach for PTSD

While studying the neural background of trauma-like behavioral deficits, we realized that NR2B subunit-expressing NMDA receptors may be involved in the behavioral deficits that result from traumatic experience. Earlier studies showed that NR2B blockers inhibit the *acquisition* of conditioned fear a frequently used model of post-traumatic stress disorder, but their effects on the *expression* of conditioned fear was poorly studied. We investigated in this experiment the effects of the selective serotonin reuptake blocker, fluoxetine, the NMDA blocker, MK-801, and the NR2B subunit blocker, Ro25-6981 on the expression of conditioned fear. Rats received 10 foot shocks administered over 5 min and were tested 24 h later in the shocking context. Treatments were administered 1 h before testing. Shocks dramatically increased freezing and reduced exploration. MK-801 and Ro25-6981 significantly ameliorated both changes. The effects of fluoxetine were less pronounced. In the open field, MK-801 increased locomotion, ataxia, and stereotypy (effects typical of NMDA blockade). Neither fluoxetine nor Ro25-6981 affected locomotion in the open field. Thus, the NR2B-specific NMDA blockade preserved the beneficial effects of general NMDA antagonists on the expression of conditioned fear but did not produce the locomotor side-effects typical of the latter. These findings warrant further studies on the effects of NR2B antagonists in models of post-traumatic stress disorder.

3.3.2. The distribution of cannabinoid ligands after intraperitoneal injections

The brain levels of cannabinoids that are reached after systemic administration are virtually unknown. To investigate this issue, we injected intraperitoneally (3)H-labeled WIN-55,212 and SR141716A (0.3, 1 and 3 mg/kg) and estimated their accumulation in the blood, adipose tissue and brain. Accumulation was dose-dependent. The largest amounts were found in the adipose tissue, while the levels seen in the blood and brain were approximately similar. The accumulation of SR141716A was markedly more pronounced than that of WIN-55,212 in all three tissues. The brain distribution of WIN-55,212 showed large regional differences. Such differences were significant but much smaller with SR141716A. The largest brain levels noticed after intraperitoneal injections did not exceed 2.5 nmol/g. This is larger than the brain level of the endocannabinoid anandamide but smaller than that of 2-arachidonoyl glycerol. Yet, the CB1 receptor affinity of WIN-55,212 and SR-141716A is two orders of magnitude larger than that of 2-arachidonoyl glycerol, suggesting that the exogenously administered compounds were functionally more active. Our findings also suggest that brain infusion and in vitro techniques employing considerably larger doses than 2.5 nmol should be dealt with caution. It appears that measuring brain levels after systemic injections increases our understanding of cannabinoid effects, and provides important clues for the comparison of results obtained with different methodologies.

3.3.3. The role of cannabinoids in addiction-related behaviors

We showed that traumatic experience induced by electric shocks durably affects behavior in tests relevant to drug abuse in conjunction with the development of post-traumatic stress disorder-like behavioral dysfunctions (see Chapter 3.1.2.4). The neural background of this

trauma-related behavioral change was not studied. However, another study of ours performed in cooperation with the group of Steve R. Goldberg (Baltimore, USA) did investigate the interaction between addiction-related behaviors and cannabinoid mechanisms (Scherma et al., 2008a). In these studies, we employed the fatty acid amide hydrolase (FAAH) inhibitor URB597 that alters endocannabinoid activity in a more functional and specific way. The inhibition of FAAH magnifies and prolongs the effects of the endocannabinoid anandamide only when and where it is synthesized and released on demand. In this study, we combined behavioral and neurochemical approaches to evaluate whether the FAAH inhibitor URB597 (cyclohexyl carbamic acid 3'-carbamoyl-3-yl ester) could alter the abuse-related effects of nicotine in rats. We found that URB597, at a dose (0.3 mg/kg) that had no behavioral effects by itself, prevented development of nicotine-induced conditioned place preference (CPP) and acquisition of nicotine self-administration. URB597 also reduced nicotine-induced reinstatement in both CPP and self-administration models of relapse. Furthermore, in vivo microdialysis showed that URB597 reduced nicotine-induced dopamine elevations in the nucleus accumbens shell, the terminal area of the brain's mesolimbic reward system. These findings suggest that FAAH inhibition can counteract the addictive properties of nicotine and that FAAH may serve as a new target for development of medications for treatment of tobacco dependence.

3.3.4. Social defeat as a new model of social phobia

We intended to use social defeat as a traumatic stressor in this project. The studies that aimed at developing this paradigm were not successful (i.e. repeated social defeat did not lead to PTSD-like behavioral dysfunctions), but interesting behavioral changes were noticed. In these studies, we repeatedly exposed rats to predictable or unpredictable psychosocial stress for 5 or 12 days and examined their anxiety in two markedly different contexts: the elevated plus maze and social interaction tests. Psychosocial stress and the social interaction test were administered under highly similar conditions, i.e. the two situations were homotypic. Psychosocial stress did not affect anxiety in the elevated plus-maze under any condition, but markedly increased anxiety in the social interaction test. In contrast, repeated restraint-a non-social stressor heterotypic to both the elevated plus maze and social interaction tests-increased plus-maze anxiety, demonstrating that anxiety in this test was sensitive to repeated restraint, and the effects were manifested in heterotypic situations. Thus, the anxiety-related effects of chronic psychosocial stress-unlike those of the chronic non-social stressor-were context-dependent. This is reminiscent of phobic anxiety, which manifests in specific situations only. In addition, behavior in the social interaction test showed changes that went beyond simple anxiogenesis. Socially stressed rats spent nearly 40% of total time in aggressive interactions. Based on recent data showing that social phobics are prone to violence under social pressure, and also based on the situation-dependent effects of the social stressor, we suggest that chronic psychosocial stress leads to a behavioral profile akin to social phobia.

3.3.5. The effects of the monoacylglicerol lipase inhibitor JZL184 in tests of anxiety

While performing the studies described above, a new compound became available namely the monoacylglicerol lipase inhibitor JZL184. Mirroring the effects of the FAAH inhibitor URB597 on brain anandamide levels, this compound enhances indirectly the brain levels of 2-arachidonoylglycerol, the other major endocannabinoid. We performed a study with this compound focusing on its anxiety-related effects (Alicki et al., 2012). The first studies with the monoacylglycerol lipase blocker JZL184 –which were performed by those who developed the compound– suggested that enhanced 2-arachidonoylglycerol signaling suppresses locomotion, lowers body temperature and decreases anxiety. Although the neurochemical effects of JZL184 develop within 30 min, its behavioral and autonomic effects were studied much later. To clarify temporal dynamics, we studied the effects of intraperitoneal JZL184 injections in mice on home-cage locomotion and body temperature for

120 min by in vivo biotelemetry. We also studied the effects of 4, 8 and 16 mg/kg JZL184 in the open-field and elevated plus-maze at various time points. In the home-cage, JZL184 blunted injection-induced body temperature increases but had no long-term effects. Vehicle injections increased the duration of rapid movements while the duration of motionless periods was decreased, a pattern also abolished by JZL184. Although the highest dose had a mild long-term effect on the relative duration of motionless periods, JZL184 seemed to have phasic rather than tonic effects in the home-cage. By contrast, open-field and plus-maze behavior was affected 80 and 120 but not 40 min after treatments, which may indicate tonic rather than phasic effects in these tests. Our findings confirm earlier reports on the mild anxiolytic effect of JZL184 but surprisingly, the compound dramatically and dose-dependently increased locomotion in the open-field in both CD1 and C57BL/6J mice. These findings are difficult to reconcile at present but suggest that the effects of MAGL inhibition are more complex than previously thought and may strongly depend on yet unidentified factors including environmental conditions, the leg time of testing, species/strains, etc.

3.3.6. Reviews

We contributed to one review summarizing the available information on the impact of the endocannabinoid system on memory and emotions (including its role in PTSD-like behavioral dysfunctions) (Zanettini et al., 2011), and wrote a review on laboratory models of anxiety, including models of PTSD (Haller and Aliczki, 2012). We incorporated in these reviews the findings and conclusions of the project reported here.

4. NEW PROJECTS EMERGING FROM THE ONE REPORTED HERE

The findings end experience accumulated during the execution of this project were used in two successful (granted) project proposals as follows:

(1) The European Research Council Advanced Grant entitled "Modulation of cortical activity by median raphe neuronal assemblies with identified behavioural effects" (Acronym: SERRACO). The principal investigator of this project is Tamás F. Freund; co-investigators are József Haller and Beáta Sperlágh. One of the important goals of the project is the study of the median raphe-cortical mechanisms that are involved in the control of PTSD-like behavioral dysfunctions.

(2) The OTKA project 101645 entitled "Trauma-induced behavioral deficits: the role of NMDA and AMPA receptor subtypes" ("Traumás élmény által előidézett magatartási zavarok: az NMDA és AMPA receptor altípusok szerepe"). This project derived from the studies presented in chapter 3.3.1.

In addition to these two projects, we intend to submit next year a project proposal for the study of the behavioral effects of the monoacylglicerol lipase inhibitor JZL184. This proposal will be based on the findings summarized in chapter 3.3.5 and additional findings obtained within the framework of other projects.

5. EXPLOITATION

Our findings suggest that the enhancement of endocannabinoid signaling by the blockade of degrading enzymes may become a treatment option for emotional disorders and possibly for the treatment of post-traumatic stress disorder.

We believe that the interaction between endocannabinoid signaling and 5-HT_3 receptor-mediated serotonergic neurotransmission –as revealed by our studies –deserves further studies as it regards its therapeutic potential in emotional disorders.

Another important finding is that the blockade of NRB2 subunit-containing NMDA receptors decreases conditioned fear a putative laboratory model of post-traumatic stress disorder.

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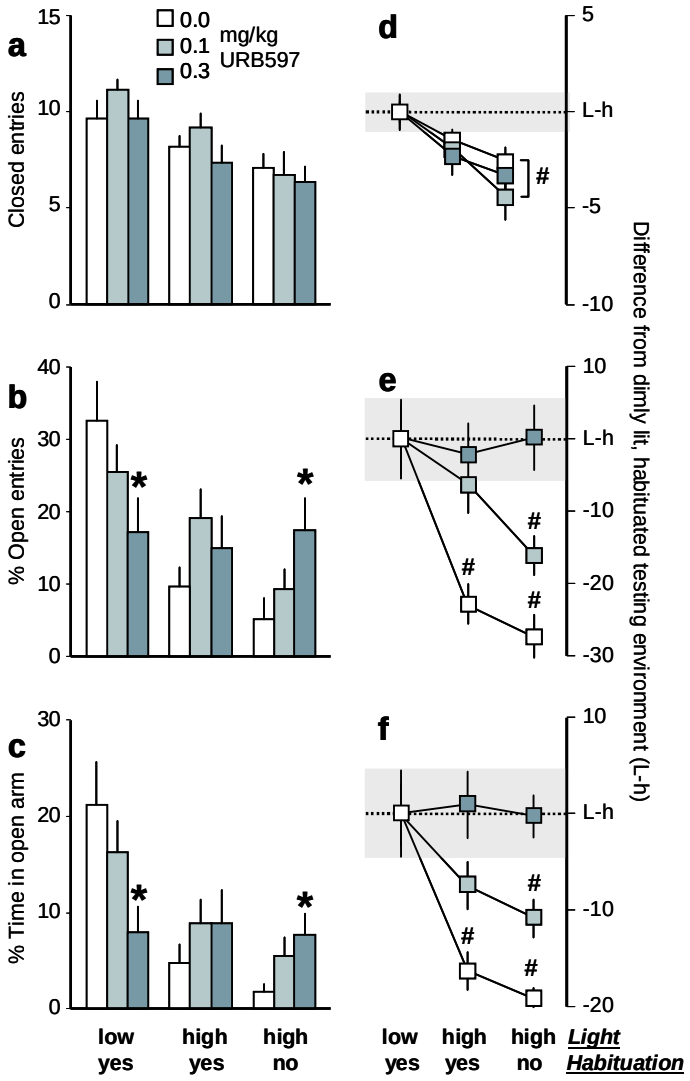

Figure 1. FAAH inhibition by URB597 eliminates the impact of light and habituation on plus-maze behavior in rats. **a-c**, closed arm entries, relative open arm entries (open/ total arm entries) and time spent in open arms, respectively, in treatment groups exposed to the elevated plus-maze under different conditions **d-e**, Changes from the low light/habituation (L-h) condition that visualize the dependence of behavior from testing conditions. Closed arm entries were significantly affected by condition only ($F_{condition}(2,110)$= 12.95; P< 0.0001), while the interaction between factors was significant for both the duration, and the relative frequency of open arm visits ($F_{treatment\ x\ condition}(4,110)$ was 3.71 (P< 0.01) and 3.59 (P< 0.01), respectively). *Grey rectangles in d-e*, s.e.m. range under L-h condition; * *and* $^{\#}$, significant effect of URB597 and condition, respectively (P< 0.05). Statistics was made on raw data; figures d-e were composed for clarity. Here and in all figures, error bars represent s.e.m.; P values underwent Bonferoni correction.

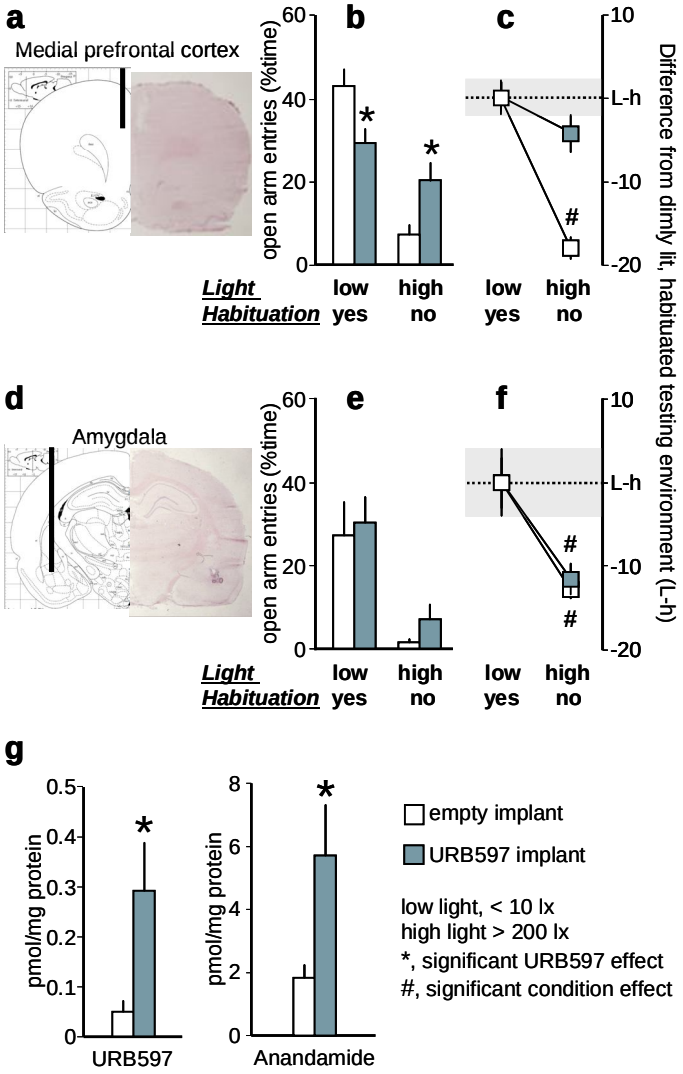

Figure 2. URB597 implanted into the prefrontal cortex but not the amygdala abolishes the effects of testing conditions in the rat elevated plus-maze. **a**, the location of URB597-containing cannullas in the prefrontal cortex. **b** and **c**, effects of prefrontal implants on anxiety-like behavior shown as in Fig. 1 a-c and d-e. **d**, the location of URB597-containing cannullas in the amygdala. **e** and **f**, effects of amygdala implants on anxiety-like behavior shown as indicated above. **g**, the concentration of URB597 and anandamide in the prefrontal cortex in sham and URB597-implanted rats, respectively. 2-archidonoil glycerol showed non-significant variation (data not shown). The effects of prefrontal implants replicated those seen after systemic injections ($F_{\text{treatment x condition}}(1,25)=$ 15.02 ($P< 0.0001$). Only the effect of condition was significant in the amygdala $F_{\text{condition}}(1,42)=$ 26.33 ($P< 0.0001$). *L-h*, low light/habituation condition used as baseline; *grey rectangles in c and f*, s.e.m. range under L-h condition; * *and* #, significant effect of URB597 and condition, respectively ($P< 0.05$). Statistics was made on raw data; figures *c and f* were composed to increase clarity.

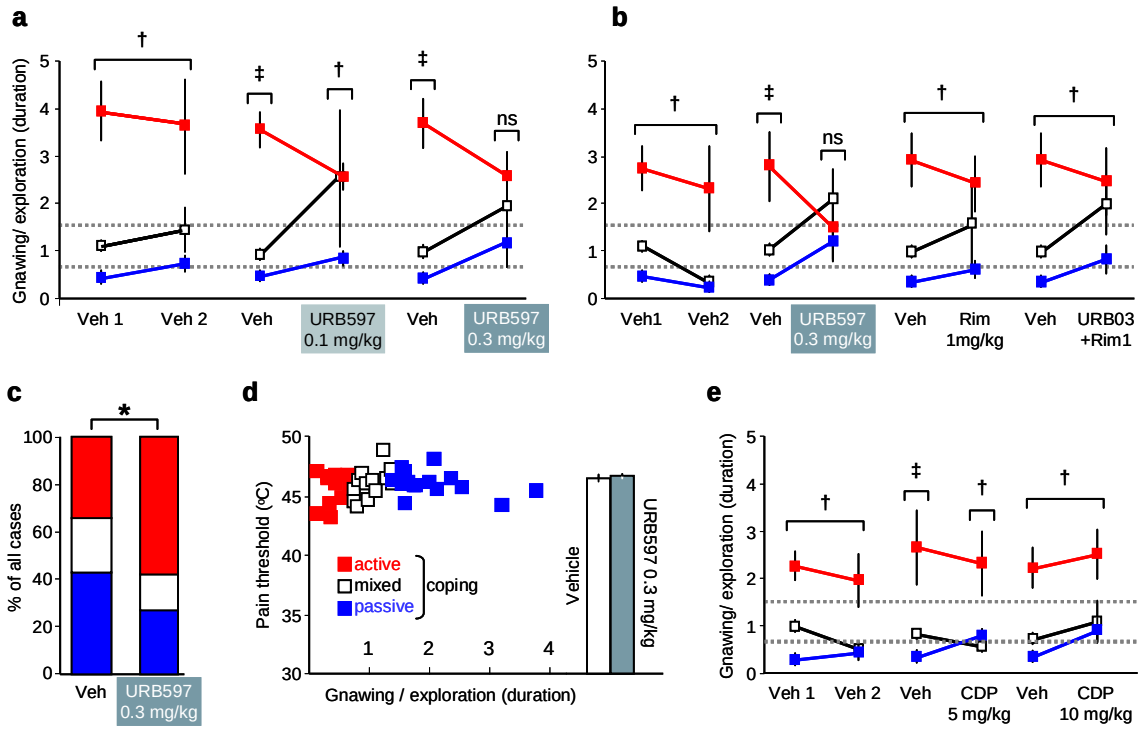

Figure 3. FAAH inhibition promotes active coping in the rat tail-pinch test. **a**, The effects 0.0 (vehicle), 0.1 and 0.3 mg/kg of URB597 on the gnawing at clamp / clamp-independent exploration ratio shown for rats showing active, mixed and passive coping styles (for further details see text and Supplementary data) ($F_{\text{strategy x treatment}}$ (2,70)= 8.00; p< 0.0001). **b**, The effects of URB597 on coping styles were abolished by the CB1 receptor antagonist rimonabant at a dose that did not affect coping styles *per se* ($F_{\text{strategy x treatment}}$ (2,68)= 5.68; p< 0.01); **c**, the distribution of strategies after vehicle and URB597 3 mg/kg treatment (Chi-Square= 11.16; p= 0.048). **d**, The impact of coping styles and URB597 on pain thresholds in the hot plate test (F(2,37)= 0.92; p> 0.4 and F(1,18)= 0.26; p> 0.6, respectively). **e**, The anxiolytic chlordiazepoxide did not close up the gap between active and passive coping. *Dotted lines*, gnawing/exploration ratios that delimited active, mixed, and passive coping styles; *Veh*, vehicle; *Rim*, rimonabant; *CDP*, chlordiazepoxide; *, significant differences in frequency distribution; ‡, all three coping styles differ significantly (P< 0.05); †, active and passive copers show significant differences; ns, no significant differences between coping styles.

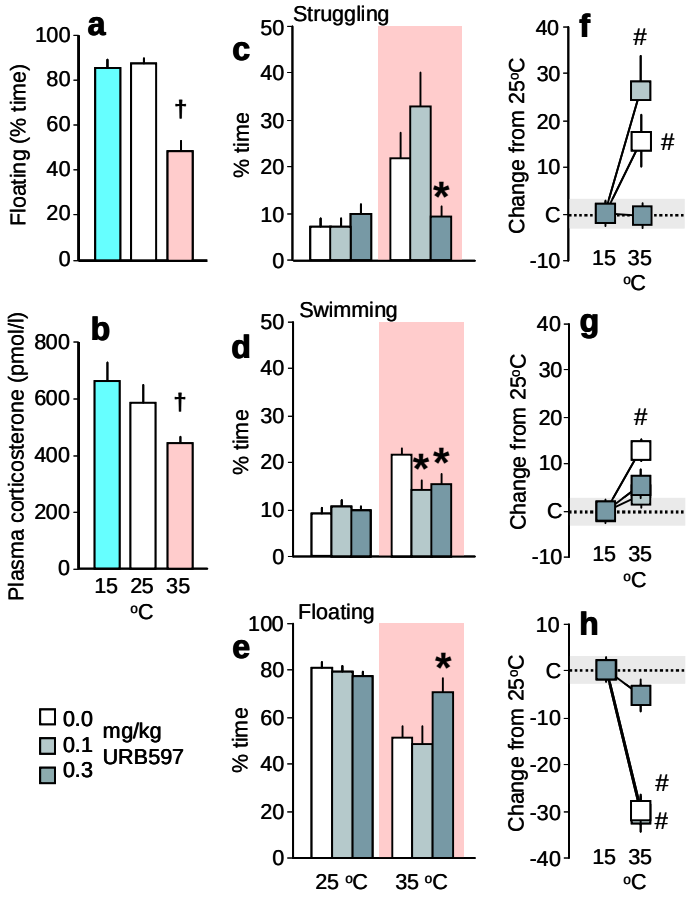

Figure 4. URB597 abolishes the behavioral effects of warm water in the mouse forced swimming test. **a**, CD1 mice float less ($F(2,27)= 30.16$; $p< 0.0001$) and **b**, show lower plasma corticosterone levels in warm as compared to cold water ($F2,32)= 4.54$; $p< 0.02$). **c-e**, URB597 did not affect behavior at 25 ^{o}C, but increases depression-like behavior at 35 ^{o}C ($F_{\text{water temperature x treatment}}= 4.11$; $p< 0.03$). Data obtained at 15^{o}C were similar to those obtained at 25^{o}C (see *Supplementary data*). **f-h**, the depression-like effect of URB597 appears to be secondary to an abolished responsiveness to water temperature. *Grey rectangles in f-h*, s.e.m. range at 25 ^{o}C; †, significant effect of water temperature; * *and* $^{\#}$, significant effect of URB597 and water temperature, respectively ($P< 0.05$). Statistics was made on raw data; figures f-h were composed to increase clarity.

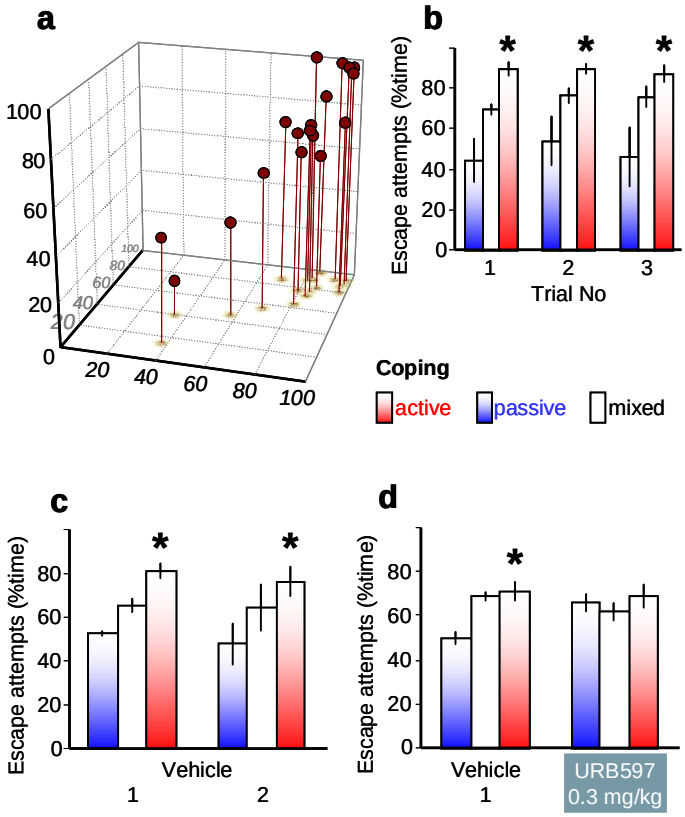

Figure 5. FAAH inhibition closes up the gap between passive and active coping in the mouse back test. **a**, three-dimensional correlation plot of escape attempts shown in three tests performed at 3 day intervals after vehicle treatments (Spearman R> 0.6; p< 0.01 at least). **b**, Coping styles observed on the first day remained stable over time in two additional testing days ($F_{coping\ style}$ (2,30)= 16.87; p< 0.001; no significant effects of trial and no interaction between factors). **c** and **d**, The effects of vehicle and URB597 treatments in a different study. Vehicle treatments did not change coping styles. In contrast, passive animals adopted an active strategy after URB597 ($F_{treatment*coping}$(2,32)= 3.42; p= 0.045). *, significant difference between active and passive animals within trials (P< 0.05).

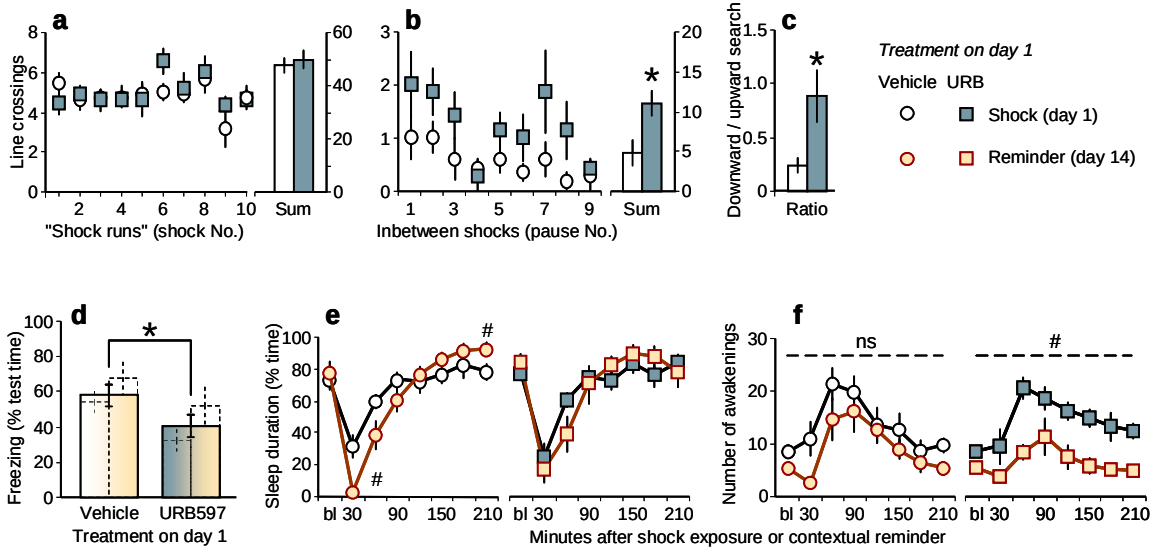

Figure 6. Ameliorative effects of URB597 on the immediate and delayed behavioral effects of electric shocks. The immediate effects of shocks on **a**, "shock-runs" (rapid locomotion directly elicited by shocks) ($F_{treatment}(1,12)= 0.15$; $p< 0.2$), **b**, locomotion during the 30 s-long brakes that separated shocks ($F_{treatment}(1,12)= 7.07$; $p< 0.03$), and **c**, the direction of exploratory behavior ($F_{treatment}(1,12)= 6.91$; $p< 0.03$). **d**, freezing was significantly affected by treatment ($F(1,12)= 5.55$; $p< 0.04$) but not by the context (shock or reminder). *Dashed columns*; freezing shown on days 1 and 14 by treatment groups; *closed columns*, the average of treatment groups. **e**, the duration of sleep ($F_{treatment*timepoint}(21,168)= 1.98$; $p< 0.01$). **f**, the number of awakenings ($F_{treatment}(3,24)= 6.15$; $p< 0.003$; $F_{timepoint}(7,168)= 18.34$; $p< 0.0001$; $F_{treatment*timepoint}(21,168)= 0.95$; $p< 0.5$). *Colored rectangles on graphs e and f*, the s.e.m. range of baselines; *, significant effect of URB597 ($P< 0.05$); $^{\#}$, significant difference between shock and reminder.