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Title: The effects anandamide signaling in the prelimbic cortex and basolateral amygdala on coping with environmental stimuli in rats

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Abstract

RATIONALE: Several lines of recent evidence suggest that endocannabinoids affect behavior by influencing the general patterns of challenge responding. **OBJECTIVES:** Here we investigated the brain mechanisms underlying this phenomenon in rats. **METHODS**: The anandamide hydrolysis inhibitor URB597 was condensed into the tip of stainless steel cannulae, which were chronically implanted slightly above the prelimbic cortex (PRL) or the basolateral amygdala (BLA), two important regions of coping and endocannabinoid action. Thereafter we investigated behavioral responsiveness to ambient light level in the elevated plus-maze and conditioned fear tests. **RESULTS:** URB597 concentration was ~30 μg/mg protein in target areas; local brain anandamide levels increased threefold, without significant changes in 2-arachidonoylglycerol. High levels of illumination halved the time spent by controls in the open arms of the plus-maze. No similar decrease was observed in rats with URB597 implants in the PRL. High light decreased conditioned fear by 30% in controls, but not in rats with prelimbic URB597 implants. Unresponsiveness to environmental challenges was not attributable to the anxiolytic effects of anandamide enhancement, as implants induced paradoxical anxiogenic-like effects under low light, which could be explained by effects on stimulus responsiveness rather than by effects on anxiety. URB597 implants targeting the BLA did not affect stimulus responsiveness. **CONCLUSIONS:** Our findings show that elevated prelimbic anandamide signaling leads to less environment-dependent (more autonomous) behavioral responses to challenges, which is an attribute of active coping styles. These findings are discussed in light of two emerging concepts of endocannabinoid roles, particularly "emotional homeostasis" and "active coping".

Keywords: amygdala, basolateral amygdala, anandamide, coping, emotional homeostasis, medial prefrontal cortex, prelimbic cortex, URB597

1. Introduction

The role of endocannabinoid signaling in anxiety is well established; it was suggested that compounds that target the endocannabinoid system hold considerable promise for the treatment of anxiety disorders either alone or as adjuncts to psychotherapy (Hill and Gorzalka 2009; Pertwee 2012; Singewald et al. 2015). However, the effects of cannabinoid ligands in laboratory tests of anxiety are often inconsistent and strongly depend on testing conditions (Moreira and Wotjak 2010; Tambaro and Bortolato 2012; Viveros et al. 2005; Zanettini et al. 2011). Several hypotheses were advanced to explain controversial effects. Moreira and Lutz (2008) identified three reasons for discrepant behavioral findings: the 'on demand' nature of endocannabinoid synthesis and release, which makes effects dependent on both environmental stimuli and internal emotional states; the wide distribution of cannabinoid receptors by which the system modulates brain regions with different functions; and finally, effects of cannabinoids on other neurotransmitters, which have opposing effects on behavior. The idea emerging from this concept is that endocannabinoids maintain emotional homeostasis by controlling neuronal excitability and by buffering the effects of environmental context and stress which may have variable consequences in particular behavioral tests (Haj-Dahmane and Shen 2011; Marco and Viveros 2009; Moreira and Lutz 2008; Morena and Campolongo 2014; Ruehle et al. 2012).

It was recently suggested that endocannabinoid signaling – in addition to, or instead of regulating particular behaviors – controls coping with environmental challenges (Haller et al. 2014; Haller et al. 2013; McLaughlin et al. 2012; Metna-Laurent et al. 2012). In our studies, coping was conceptualized according to Koolhaas et al. (1999) who defined active and passive coping styles as distinct behavioral phenotypes, which differ in patterns of responding upon challenge. Active copers base their behavior on routines that are weakly influenced by environmental stimuli (i.e., they show low behavioral responsiveness) and attempt to control challenges when they occur, whereas passive copers are governed by environmental stimuli (are behaviorally more responsive) and respond challenges by avoidance behavior. We showed that single systemic treatments with the fatty acid amide hydrolase (FAAH) inhibitor URB597 (which enhances the brain levels of the endocannabinoid anandamide by inhibiting its hydrolysis) promotes active coping in both mice and rats. Subjects treated with this agent were less responsive to minor changes in the environment (e.g. the level of illumination), and focused their behavior on controlling major disturbing factors e.g. they actively tried to remove a clamp attached to their tail, or struggled more when forced on their

backs in the backtest (Haller et al. 2014; Haller et al. 2013). These findings together with those obtained in other laboratories resulted in the "coping theory" of cannabinoid action, which is congruent with the "emotional homeostasis theory", but adds a new dimension to the latter and may have particular therapeutic implications (see Discussion).

The aim of the present studies was to investigate the brain sites where cannabinoids affect coping with challenges, particularly responsiveness to environmental conditions, which is one of the major attributes of coping styles (Koolhaas et al. 1999). We addressed long-term interactions between coping and endocannabinoid signaling, by chronically implanting the anandamide hydrolysis blocker URB597 in the prelimbic area of the prefrontal cortex (PRL) and the basolateral amygdala (BLA), two brain regions involved in the control of all three coping, stress responses, and anxiety (Coppens et al. 2010; Koolhaas et al. 2010). URB597 was implanted by a technique developed earlier by our group (Barna et al. 2007), where chronic implants released cannabinoid ligands poorly soluble in water for extended periods, and the release was restricted to the implanted brain region.

Rats were studied one week after implantation in the elevated plus-maze under high and low illumination. This experiment investigated the degree to which the behavior of rats was influenced by environmental conditions, i.e. it tested behavioral responsiveness to environmental stimuli. In addition, we studied the effects of URB597 implanted into the PRL or the BLA on contextual conditioned fear and the impact of test-cage illumination on this response. This experiment evaluated the brain area-specific role of anandamide signaling on coping with a major and a minor environmental stimulus i.e. the fear-associated context and a less relevant component of this context i.e. light intensity.

2. Experimental Procedures

2.1. Subjects

Subjects were 2–3 months old male Wistar rats (Charles River Laboratories; Hungary) weighing approximately 250 g. Food and water were available *ad libitum*; temperature and relative humidity were kept at 22 ± 2 °C and 60 ± 10%, respectively. Subjects were maintained in a normal light cycle of 12 h with lights off at 07:00 h. Acclimatization to local conditions lasted at least one week. Subjects were kept in groups of 4 in polycarbonate cages 45 × 35 × 25 cm. All subjects were experimentally naive with no drug history prior to the study. Each experiment was conducted in a different set of subjects; each animal was used in one experiment only.

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 our Institute.

2.2. Experimental design

In *Experiment 1* we assessed whether implants of URB597 crystals are able to locally increase the levels of anandamide and 2-AG in the brain. The prefrontal cortex was in the focus of this study because earlier findings showed that single or repeated URB597 treatments increase anandamide levels strongly and specifically in the amygdala but not in the prefrontal cortex (Bortolato et al., 2007; Lomazzo et al., 2015). However, the effects of chronic local URB597 implants were not investigated earlier. One week after the implantation of URB597 or empty cannulae into the PRL, rats were terminated by decapitation, and their brains were sampled for HPLC measurements. Sample size was 8 per group.

In *Experiment 2* we studied the effects of URB597 implanted into the PRL on behavior shown in the elevated plus-maze, which has been proposed to measure anxiety-like and risk assessment behavior. The test was run either under low or high light conditions (5 lx or 300 lx, respectively). The procedure was similar to that employed when the coping-related effects of singly systemic URB597 treatments were studied (Haller et al. 2013). Briefly, rats were transferred together with their home-cages into the testing room and were habituated to testing light intensity (5 or 300 lx) for 60 min. After habituation, they underwent 5 min of testing on the EPM under the respective illumination condition. Sample size was 7-8 per group.

In *Experiment 3* we studied the effects of URB597 implanted into the PRL in the conditioned fear test. One week after implantation, animals either underwent fear conditioning or were placed in the apparatus without conditioning. Rats were tested for conditioned fear 24h later. Rats were studied either under low or high light (5 lx or 300 lx, respectively). Sample size was 10-12 per group.

In *Experiment 4 and 5* rats were studied as described for *Experiments 2* and *3*, but implants targeted the BLA this time. Sample size was 10-12 per group for *Experiment 4* and 9-12 subjects per group for *Experiment 5*.

2.3. The implantation of URB597 into discrete brain areas

We employed the method developed earlier by our group (Barna et al. 2007), which was based on the procedure of Grossman (Grossman 1960). Crystals of URB597 (Sigma, Budapest,

Hungary) were pulverized mechanically and condensed into the tip of stainless steel cannulae (25 G injection needle, Medicor, Hungary) by gentle pressure. The length and the outer/inner diameters of the cannulae were 9 and 0.5/0.2 mm, respectively. The outer surface of cannulae was cleaned. Cannulae for control rats were empty. Rats were anesthetized by a mixture of ketamine, xylazine, and pipolphen (50, 10, and 5 mg/kg, respectively) and the head was fixed into a stereotaxic frame (flat skull position). Two cannulae were implanted bilaterally. For experiments addressing the role of the PRL, the tip of the cannula was targeted towards the dorsal region of this area (coordinates: 2.5 mm anterior to the Bregma, 0.7 mm lateral to the midline, and 3.0 mm from the dura). For experiments addressing the role of the BLA, the tip of the cannulae was also targeted towards the upper region of this area (coordinates: -2.6 mm posterior to the Bregma, 4.8 mm lateral to the midline, and 7.5 mm from the dura). Implantation coordinates were based on the atlas of Paxinos and Watson (1998). The cannulae were fixed to the skull by jeweler's screws and dental acrylic. After surgery, rats were maintained in individual cages. Following behavioral experiments, the location of the cannulae was checked by histological methods. Cannulae were also checked for their content. All rats included in our studies had appropriately placed cannulae, the tip of which still contained residuals of the compounds at the end of experiments. This shows that the URB597 was not removed during the surgery and was available throughout the experiment. In addition to this visual inspection, the brain content of URB597, anandamide and 2-AG was measured by HPLC in the prefrontal cortex for the reasons shown above.

2.4. Behavioral testing and analyses

Behavioral tests were conducted one week after implantation, in the early hours of the light phase in a separate quiet room. Behavior was video recorded in all tests by a SonyDCR-SR75 digital video camera. Video recordings were analyzed with the custom-made H77 event recording software (J. Haller, Institute of Experimental Medicine, Budapest, Hungary). Behavior was always scored by an experimenter blind to treatments. Animals belonging to the same study were scored by the same observer. Intra-rater reliability was over 90%.

The elevated plus-maze was made of dark gray painted wood (arms: 50 * 17 cm, wall height: 30 cm; platform height: 80 cm). Subjects were placed in the central area of the apparatus with head facing a closed arm. Exposure lasted 5 min. The number of entries into the closed arms (expressed as counts/5 min) was considered an indicator of locomotor activity, whereas open arm

exploration as an indicator of anxiety (Pellow et al. 1985). This was characterized by two variables: the duration of open-arm visits (expressed as % of total time), and % open-arm entries (open-arm entries/total arm entries×100). Both correlate negatively with anxiety. We also evaluated risk assessment behavior, particularly stretch attend posture (the rat standing in a stretched posture and investigating the environment) and head dipping (the rat investigating the area beneath the plus-maze) from protected areas of the elevated plus-maze (i.e. from the closed arm and the central platform); both correlate positively with anxiety (Rodgers and Johnson 1995).

Fear conditioning and testing. On the first day of the protocol, rats were introduced into a Plexiglas cage of 30 * 30 * 30 cm, where they received electric shocks *via* the grid floor of the box. Two trains of shock pulses of 1 s were administered per minute for 5 min (i.e. each rat received 10 shocks). Each shock train (100 V, 3mA) was 1 s long and consisted of 0.01 sec shocks separated by 0.02 sec-long breaks. The box was cleaned after each animal with ethanol. After fear conditioning, rats were returned to their home cages and were re-exposed to the shock-associated cage 24h later for 5 min. Shocks were not delivered this time. Behavior was video recorded and scored later by an experimenter blind to the treatments. We recorded the time spent in immobility (no movements other than respiration), which indicates contextual fear.

2.5. The measurement of endocannabinoid levels in the brain

One week after the implantation of URB597, rats were terminated by decapitation; their brains were quickly removed and dissected into 1 mm thick brain slices on an ice-cold plate using a stainless steel rat brain blocker (RBM-4000C, ASI Instruments, Texas, USA). In two sections surrounding the tips of the cannulae, we dissected a 2 * 2 mm sample immediately below the tip of the cannula. The sample was frozen on dry ice and stored at -70^{o}C till further analysis. The weighed frozen tissue was homogenized in an appropriate volume of ice-cold 0.1 M perchloric acid. The suspension was centrifuged at 300 g (4500 rpm) for 10 min at 4 ^{o}C. The excess of perchlorate anion of the supernatant was precipitated by addition of 2 M KOH and was removed by centrifugation for 10 min at 4^{o}C. The supernatant was stored at -20^{o}C until analysis. The pellet was saved for protein measurement (Lowry et al. 1951). Pre-column derivatization was performed by mixing 100 µl of tissue supernatant and 150 µl of 2 M sodium carbonate that contained 20 µM norvaline as internal standard, and 150 µl of dansyl chloride that was prepared by daily dissolving 5 mg 5-(dimethylamino)-1-naphthalenesulfonyl chloride in acetonitrile. After 10 min reaction time at 60^{o}C the mixture was acidified with 100 µl 6 M formic acid, was chilled

and 400 µl of the aliquot was injected onto the enrichment-column. We used a Gilson liquid chromatographic System with 715-operation software (Gilson Medical Electronics inc., Middletown, and WI USA). Two delivery pumps (Model 305) and a programmable auto injector (SIL-10AD, Shimadzu) were built in. The online enrichment was performed on a 3 µm Eurosphere C18 (100 * 2.1) column, with analytical mobile phase „A" at 0.2 ml/min flow rate for 4.5 min. Dansylated anandamide and other compounds of the tissue extract were separated on a 3 µm Discovery C18 HS (250 * 2.1 mm) analytical column. The mobile phase was 15 mM ammonium formate, pH 3.7; the methanol-acetonitrile content was 5 % and 90 % (V/V) in buffer „A" and „B", respectively. URB597 and anandamide were detected by the absorbance detector Agilent 1100 adjusted to 319 nm. The dansylated derivatives were detected using a fluorescence detector set at 310-410 nm excitation, and 480-520 nm emission wavelengths. Concentrations were calculated by the two-point calibration curve method. The data were expressed in pmol per mg protein.

2.6. Statistical analyses

Data are presented as mean ± standard error of the mean. Two-way analysis of variance (ANOVA) was used to estimate main effects. Post-hoc Duncan tests were performed only for significant main effects. Behavioral data were square root-transformed when ANOVA assumptions were not fulfilled. Assumptions were checked before and after transformation by the Levene test. Significance level was set at $P < 0.05$. P values for multiple comparisons underwent Bonferroni correction (Holm's procedure).

3. Results

3.1 The effects of implants on brain endocannabinoid levels (Experiment 1)

The concentration of URB597 was 28.4 ± 12.3 µg/mg protein in the target area, whereas its levels in the hippocampus and striatum were around the detection limit (2.4 ± 0.7 and 2.5 ± 1.3 µg/mg protein, respectively). URB597 implants significantly increased anandamide levels in the PRL ($F(1,14) = 5.25$, $p = 0.038$; Fig. 1a,b). 2-AG levels did not significantly differ between sham and URB597 implanted rats.

3.2. Behavioral effects of URB597 implanted into the prelimbic cortex (Experiment 2 and 3)

The cannulae were correctly placed in all rats in Experiment 2 and 3 (Fig. 2). In the elevated plus-maze test, closed arm entries were unchanged by treatments or illumination (Fig. 3a). By contrast, open arm activity was affected by the interaction between the illumination of the

testing environment and treatment (*ratio of open arm entries*: $F_{illumination*treatment}(1,25) = 9.41$; $p < 0.01$; *time in open arms*: $F_{illumination*treatment}(1,25) = 20.39$; $p < 0.01$) (Fig. 3a). Ostensibly, URB597 implants increased anxiety under low light but decreased it under high light. These differences, however, resulted from light-induced changes in the behavior of controls, which showed considerably more anxiety under high than under low light. URB-implants abolished this difference. A similar situation was observed with risk assessment behaviors, which show a positive correlation with anxiety. The duration of stretch attend posture shown under low and high light were dramatically different in controls (1.13 ± 0.54 and 3.41 ± 0.5, respectively), whereas illumination did not affect this behavior in URB597-treated rats (1.43 ± 0.4 and 1.16 ± 0.5, respectively). In a similar fashion, the duration of head dipping from protected areas depended on illumination in controls (*low light*: 1.69 ± 1.2; *high light*: 4.67 ± 0.4), but not in URB597-implanted rats (*low light*: 2.44 ± 0.5; *high light*: 2.36 ± 0.5). Taken together, these findings show that the behavior of controls did, whereas the behavior of URB597-implanted rats did not depend on environmental stimuli.

In the conditioned fear test, the duration of immobility was dramatically increased in fear conditioned as compared with non-fear conditioned rats ($F_{footshock}(1,77) = 183.04$; $p < 0.01$) (Fig. 3b). In non-conditioned controls, the duration of immobility depended on illumination ($F_{illumination}(1,39) = 13.85$; $p < 0.01$) but not on treatment. In fear conditioned rats, immobility showed a tendency to depend on the interaction between factors ($F_{illumination*treatment}(1,38) = 3.54$; $p = 0.06$), and post-hoc comparisons showed that high light significantly decreased the time spent in immobility in rats with empty cannulae, but not in URB597-implanted rats. Taken together, these findings show that URB597 did not alter the effects of fear conditioning, but affected the impact of environmental stimuli on behavioral responses.

3.3. Behavioral effects of URB597 implanted into the basolateral amygdala (Experiment 4 and 5)

The cannulae were correctly placed in all rats in Experiment 4 and 5 (Fig. 4).In the elevated plus-maze, illumination had no effect on closed arm entries, while its high levels decreased both the ratio of open arm entries ($F_{illumination}(1,41) = 52.78$; $p < 0.01$) and time spent in the open arms ($F_{illumination}(1,41) = 37.12$; $p < 0.01$) (Fig. 5a). URB597 implantation caused no changes in the above variables (Fig. 5a). Changes in stretch attend postures were highly similar. Low light decreased the duration of this behavior (indicating lower levels of anxiety), whereas the effects of URB597 implants were not significant, albeit lower values were obtained under both light

conditions (*controls, low light*: 0.84 ± 0.48; *URB597, low light*: 0.51 ± 0.24; *controls, high light*: 3.00 ± 0.41; *URB597, high light*: 2.43 ± 0.29) ($F_{illumination}$ (1,39) = 43.85; p = 0.0001). The duration of head dipping from protected areas showed no changes induced by illumination or URB597 implants (*low light*: 1.69 ± 1.2; *high light*: 4.67 ± 0.4), but not in URB597-implanted rats (*controls, low light*: 2.23+0.5; *URB597, low light*: 1.09+0.25; *controls, high light*: 2.46+0.59; *URB597, high light*: 2.05+0.4).

Fear conditioning significantly increased immobility in the conditioned fear test ($F_{footshock}$ (1,77) = 95.84; p < 0.01) (Fig. 5b). The increase -although highly significant- was smaller than that seen in Experiment 3 where implants were aimed at the PRL. Such variations in behavior (i.e. behavioral differences among control animals across experiments) are not uncommon among laboratory rodents, especially outbred lines such as Wistar rats. One cannot rule out, however, that in addition to such differences, the discrepancy was partly due to damages made to structures located dorsally to the BLA, e.g. the lateral amygdala. However, the target region BLA suffered no damages. In rats not exposed to fear conditioning, immobility depended on the illumination of the testing environment ($F_{illumination*treatment}$ (1,41) = 8.47; p< 0.01) but not on treatment. In rats exposed to fear conditioning, no group differences were observed.

4. Discussion

4.1. Main findings

A mechanical approach to plus-maze findings suggests that prelimbic URB597 implants increased anxiety under low but decreased anxiety under high light. In fact, however, URB597 eliminated the effects of illumination: URB597 implanted rats spent about 25% of their time in the open arms irrespectively to light conditions, while control subjects showed a marked avoidance of the open arm in high light. The conditioned fear test showed that prelimbic URB597 implants do not affect fear memory retrieval, but again: the behavior of controls did, while the behavior of implanted rats did not depend on illumination. The implants did not interfere with vision, as the impact of light on immobility was similar in non-shocked controls irrespective to their treatment. Taken together, the behavior of implanted rats was less responsive to environmental conditions, and was more focused on task-specific behaviors: the exploration of an unfamiliar arena and defensive behavior in a dangerous context. URB597 implants in the BLA did not affect behavior, suggesting that behavioral responsiveness to environmental changes is specifically affected by prelimbic anandamide signaling.

4.2. Comparisons with earlier findings and putative mechanisms

Acute prefrontal injections of the cannabinoid agonists Δ9-tetrahydrocannabinol and methanandamide as well as the FAAH inhibitor URB597 affect behavior in the elevated plus-maze in a biphasic manner: low doses are anxiolytic, whereas high doses are anxiogenic (Rubino et al. 2008a; Rubino et al. 2008b). Somewhat more complex findings were obtained by Fogaca et al. (2012) with the cannabinoid agonist ACEA, which was anxiolytic at 5 pmol/kg, and non-effective at a higher dose (50 pmol/kg); however, the higher dose became anxiogenic when combined with the CB1 receptor antagonist AM251 and anxiolytic when combined with a TRPV1 blocker. These findings – in line with those obtained by Rubino et al. (2008b) – suggest that anxiogenic effects resulting from prefrontal cannabinoid treatments are mediated by the TRPV1 receptor. In all these studies, rats were tested under dim light; as such, the anxiogenic effects of larger doses correspond to the anxiogenic effects of URB597 implants under low light in our study i.e. are consistent with our findings. At the same time, they raise the possibility that ostensible anxiolytic and anxiogenic effects observed under different levels of illumination were due to an interaction between the CB1-mediated and TRPV1-mediated effects of enhanced anandamide signaling, and tentatively suggest a dynamic ability for anandamide signaling to rapidly flip between signaling pathways in response to environmental cues. Earlier studies, however, do not support this assumption. Firstly, systemic URB597 treatments resulted in effects highly similar to those obtained here with prefrontal URB597 implants, and these effects were abolished by the CB1 antagonist AM251 (Haller et al. 2013). Secondly, TRPV1 receptors –in contrast to CB1 receptors– appear to be downregulated by the chronic enhancement of anandamide signaling; moreover, the antinociceptive effects of chronic FAAH inhibition were attributed to the desensitization of this receptor (Moreira et al. 2012; Ross 2003; Schlosburg et al. 2010; Starowicz and Przewlocka 2012). These considerations suggest that the effects reported here were primarily mediated by the CB1 and not by the TRPV1 receptor. However, the mechanisms underlying the URB597-induced changes in stimulus responsiveness certainly require further studies.

Endocannabinoid signaling appears to promote fear extinction without major effects on fear retrieval (*CB1-KO mice*: (Marsicano et al. 2002); *the FAAH inhibitor AM3506*: (Gunduz-Cinar et al. 2013); *the anandamide reuptake inhibitor AM404*: (Chhatwal et al. 2005)). These findings are in line with ours, where the chronic implantation of URB597 into the medial prefrontal cortex did not affect fear retrieval.

Surprisingly, BLA implants did not affect behavior in our study, despite the fact that the amygdala as a whole is considered to be among the main brain areas that control anxiety and where cannabinoids exert anxiolytic effects (Aliczki and Haller 2015; Davidson 2002; Davis 1992). However local cannabinoid treatments targeting specifically the BLA provided ambiguous findings: the cannabinoid agonists THC and WIN55,212-2 affected plus-maze anxiety and conditioned fear under specific conditions only (e.g. after the co-application of other stressors or at low dosage only; (Ganon-Elazar and Akirav 2009; Rubino et al. 2008a), whereas acute local URB597 injections had no reliable effects on plus-maze behavior, albeit they decreased anxiety when TRPV1 receptors were also blocked (John and Currie 2012). Recent findings suggest that enhanced anandamide signaling in the BLA affects the storage of fear memories rather than their retrieval (Kamprath et al. 2011; Munguba et al. 2011), which is consistent with our findings with removable URB597 implants (Barna et al., unpublished data). Taken together, these findings suggest that the BLA anandamide signaling has a relatively low contribution to the anxiety-related roles of the amygdala. Considering, however, its roles in coping, BLA implants could still affect stimulus responsiveness in our study, which did not happen. It is unlikely that this was due to technical reasons, as the PRL and BLA show no large differences in size, which would have affected the extent to which URB597 penetrated them, and the BLA was not lesioned by implants. Local features of cannabinoid signaling do not show major differences either; e.g. the expression of FAAH and CB1 receptors is rather similar in the two structures (Herkenham et al. 1991; Thomas et al. 1997; Tsou et al. 1998). Taken together, earlier and present findings suggest that anandamide signaling in the BLA affects anxiety under certain conditions, but does not mediate the impact of environmental stimuli on the expression of fear.

4.3. Cannabinoids and coping

An emerging new concept suggests that endocannabinoid signaling influences coping styles, and raises the possibility that the context-dependence of cannabinoid effects in various behavioral tests is a reflection of this more general impact on behavior. This idea was forwarded by several authors. McLaughlin et al. (2012) showed that acute systemic treatments with the anandamide hydrolysis inhibitor URB597 decreases immobility and increases swimming in the forced swimming test of depressive-like behavior i.e. enhances active responses on the expense of passive responses. Metna-Laurent et al. (2012) showed that conditional CB1-KO mice show a decrease in active coping in the conditioned fear test as indicated by a delayed return to normal activity after

exposure to fear-associated cues. We have shown that rats treated with URB597 disregard environmental information (e.g. light intensity) in the elevated plus-maze, and intensify their efforts to remove a slightly painful clamp from their tail (Haller et al. 2013). These changes were seen after acute systemic treatments, and were interpreted as decreases in behavioral flexibility and increases in active "problem solving" which are the two principal attributes of the active coping style (Koolhaas et al. 1999). These findings were supported later by experiments performed in mice (Haller et al. 2014). It is worth to note that decreased behavioral flexibility does not necessarily reflect a detrimental change in behavior, and may also be attributed to increased focus under challenging situations. For instance, the two experimental situations studied here involve a component of a major, and another of a more minor importance. The "main task" in the elevated plus-maze is to explore an anxiety-provoking novel environment, whereas in the conditioned fear test the main task is to avoid a danger by immobility, an evolutionarily-shaped response that decreases visibility under distant threat (Baldwin 2013). Disregarding less important environmental stimuli (e.g. light intensity) under conditions of threat reflects decreased distractibility (focused attention) rather than a behavioral deficit.

Regarding brain mechanisms, McLaughlin et al. (2012) demonstrated that active behavioral responses in the forced swimming test of depression are controlled by an acute increase in prefrontal endocannabinoid signaling; Metna-Laurent et al. (2012) showed that active coping with an anxiety-provoking situation is controlled by cortical glutamatergic neurons, while the present findings show that chronic increases prefrontal endocannabinoid signaling decreased distractibility under conditions of challenge. Taken together, these findings show that endocannabinoids promote various aspects of active coping style by influencing cortical, primarily prefrontal function.

The relationship between emotional homeostasis and coping styles, the two "general theories" of endocannabinoid action, is probably intricate. Emotional homeostasis was explained as "avoiding excesses and deficiencies" (Marco and Viveros 2009), "an appropriate reaction to stressful events" (Ruehle et al. 2012), and as "an emotional buffer" (Morena and Campolongo 2014). Active coping on its turn is an extreme of a continuum (the other extreme being passive coping), and may not always constitute an "appropriate response" (Koolhaas et al. 1999). For instance, focusing on relevant cues by disregarding less relevant ones seemed to be a favorable change under the conditions of our study (see above), but it may be the reason why enhanced anandamide signaling interferes with reversal learning in the attentional set shift task (Klugmann

et al. 2011). It was posited that active coping is advantageous in certain situations only (Koolhaas et al. 1999); it occurs that decreased distractibility as a symptom of active coping is advantageous when dangers are to be averted, but disadvantageous when learning needs to be re-evaluated.

Based on the above, the phrases "emotional homeostasis" and "active coping" appear to capture different aspects of behavior. Nevertheless, emotional homeostasis may be a prerequisite of active coping: it may allow the subject to deal with challenges actively, and to focus its attention on relevant cues. Further insights into the roles of endocannabinoids in emotional homeostasis, active coping and their relationship may open a new window for therapeutic interventions targeting the cannabinoid system, because coping is a reliable predictor of disease-induced decreases in quality of life, and interventions promoting active coping styles – which are associated favorably with resilience – have been proposed as therapeutic goals for a variety of physical diseases and mental disorders (Cooke et al. 2007; Pucheu et al. 2004; Tiemensma et al. 2011; Westerhuis et al. 2011).

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Figure legends

Figure 1 The effects of URB597 implants on anandamide (AEA) and 2-arachidonoylglycerol (2-AG) content in the 2 * 2 * 1 mm section (dark gray square in the right panel) dissected below the tip of the implantation cannula (black line in the right panel) (the experimental design is shown above the results). This study was undertaken because earlier studies suggested a weak effect of URB597 in the prefrontal cortex (Bortolato et al., 2007; Lomazzo et al., 2015). As earlier studies showed that the compound strongly and specifically increases AEA levels in the amygdala, this brain region was not investigated. *HPLC*, brain sampling for the measurement of endocannabinoid levels by high-performance liquid chromatography (HPLC); *IL*, infralimbic cortex; *PRL*, prelimbic cortex; *PRL URB597*, the bilateral implantation of URB597-containing or empty cannulae in the prelimbic cortex (PRL); *black line*, cannula aiming at the PRL.

Figure 2 The placement of cannula tips targeting the prelimbic cortex in Experiment 2 and 3 (the schematic was adapted from Paxinos and Watson, 1998). A representative photomicrograph of cannula placement is adjacent to schematic diagrams. *CFT*, conditioned fear test; *EPM*, elevated plus-maze test (performed in a different set of rats); *fmi*, forceps minor; *IL*, infralimbic cortex; *PRL*, prelimbic cortex

Figure 3 The effects of URB597 implants targeting the prelimbic cortex (PRL). **a**. the experimental design and the findings of the elevated plus-maze study; **b.** the experimental design and the findings of the conditioned fear study. For risk assessment findings see text (section 3.2 and 3.3). *, significant effect of URB597 compared to controls tested under similar light conditions; #

significant effect of light ($p < 0.05$ after Bonferroni correction). *CFT*, conditioned fear test; *EPM*, elevated plus-maze test (performed in a different set of rats); *FC*, fear conditioning; *PRL URB597*, the bilateral implantation of URB597-containing or empty cannulae in the PRL.

Figure 4 The placement of cannula tips targeting the basolateral amygdala in Experiment 4 and 5 (the schematic was adapted from Paxinos and Watson, 1998). A representative photomicrograph of cannula placement is adjacent to schematic diagrams. *BLA*, basolateral amygdala; *BLA URB597*, the bilateral implantation of URB597-containing or empty cannulae in the BLA; *BMA*, basomedial amygdala; *CeA*, central amygdala; *CFT*, conditioned fear test; *EPM*, elevated plus-maze test (performed in a different set of rats)

Figure 5 The effects of URB597 implants targeting the basolateral amygdala (BLA). **a**. The experimental design and the findings of the elevated plus-maze study; **b**. the experimental design and the findings of the conditioned fear study; **c**. *BLA URB597*, the bilateral implantation of URB597-containing or empty cannulae in the BLA; *CFT*, conditioned fear test; *EPM*, the elevated plus-maze test; *FC*, fear conditioning; # significant effect of light ($p< 0.05$ after Bonferroni correction).

Fig. 1.

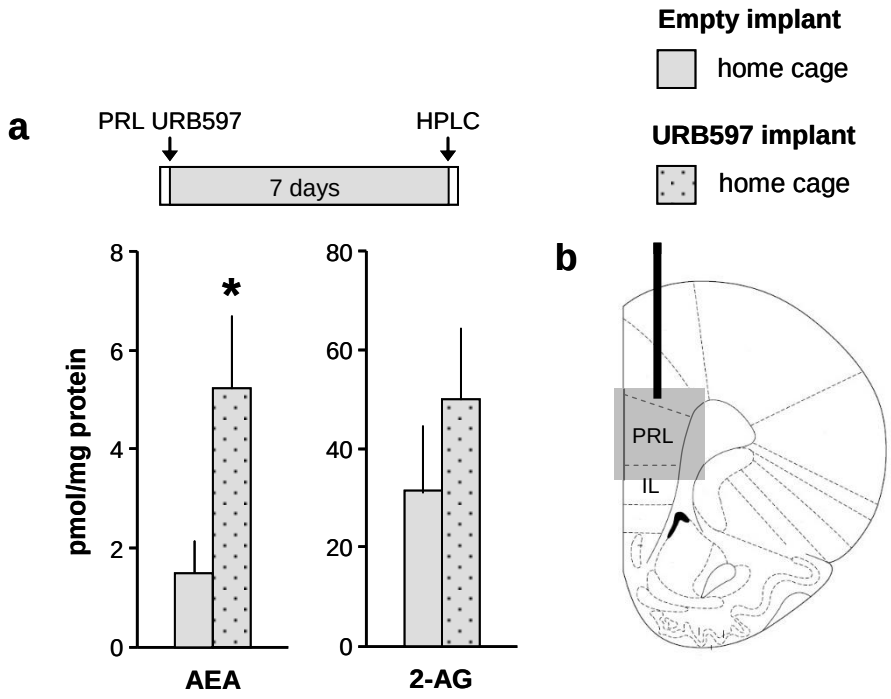

Empty implant
home cage
URB597 implant
home cage
a
PRL URB597
HPLC
7 days
pmol/mg protein
8
6
4
2
0
*
80
60
40
20
0
AEA
2-AG
b
PRL
IL

Fig. 2.

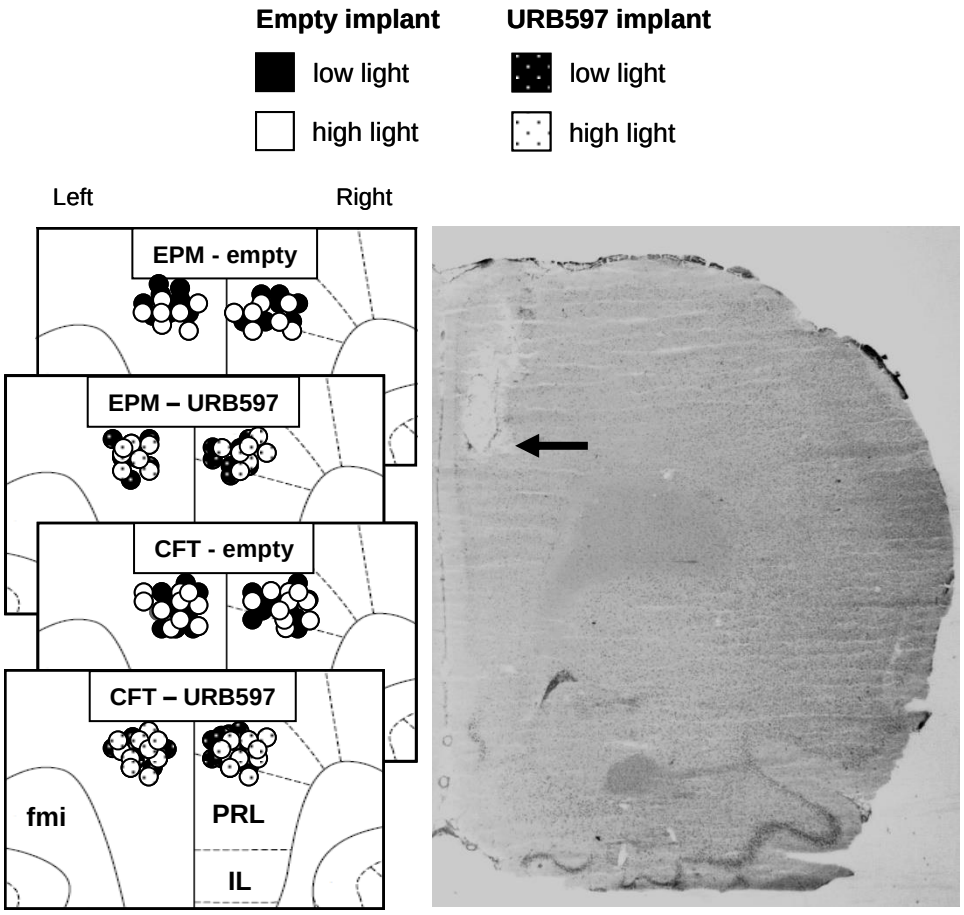

Empty implant
URB597 implant
low light
high light
low light
high light
Left
Right
EPM - empty
EPM – URB597
CFT - empty
CFT – URB597
fmi
PRL
IL

Fig. 3

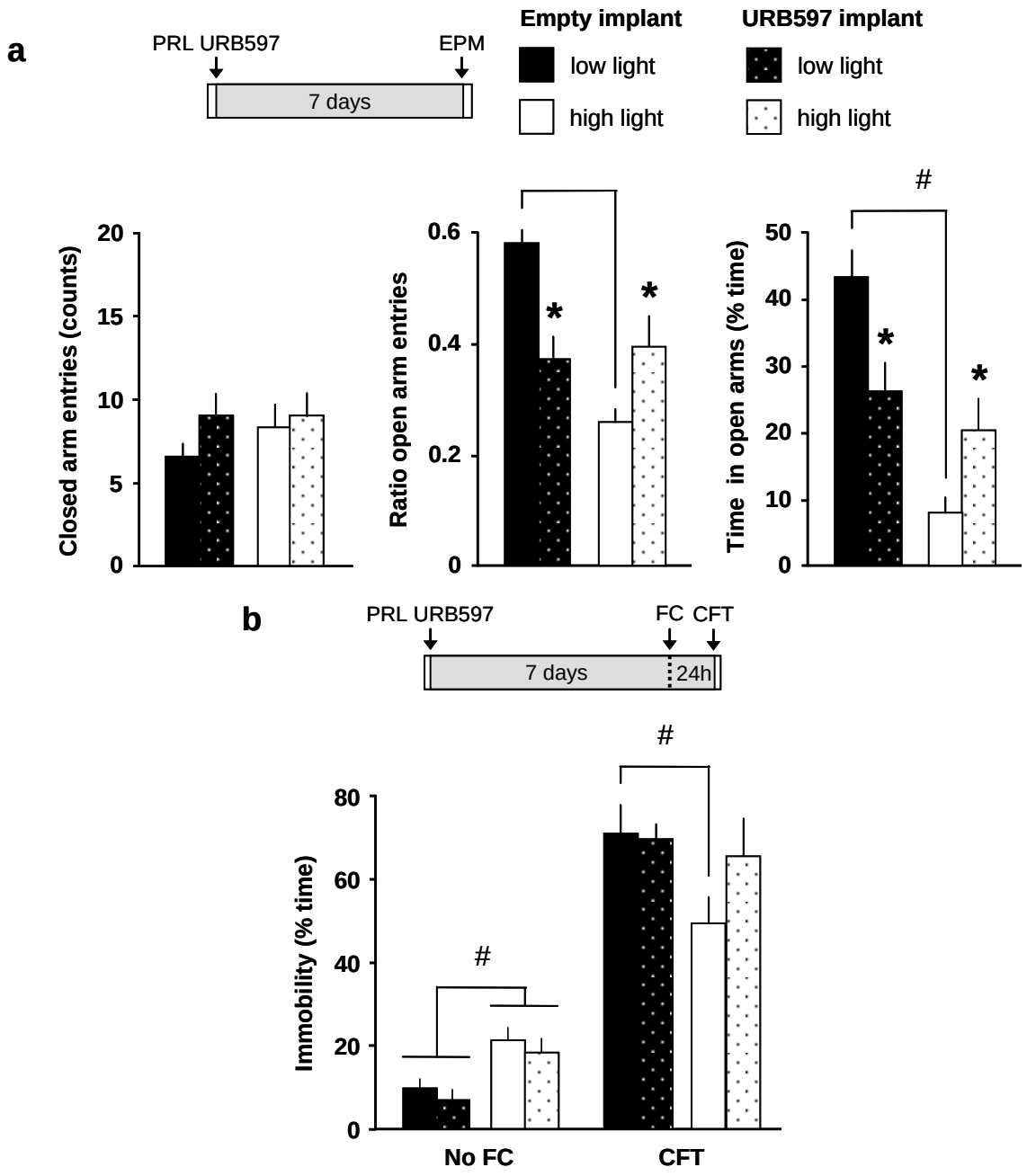

a
PRL URB597
EPM
7 days
Empty implant
low light
high light
URB597 implant
low light
high light
Closed arm entries (counts)
Ratio open arm entries
Time in open arms (% time)
#
*
b
PRL URB597
FC CFT
7 days
24h
Immobility (% time)
No FC
CFT

Fig. 4

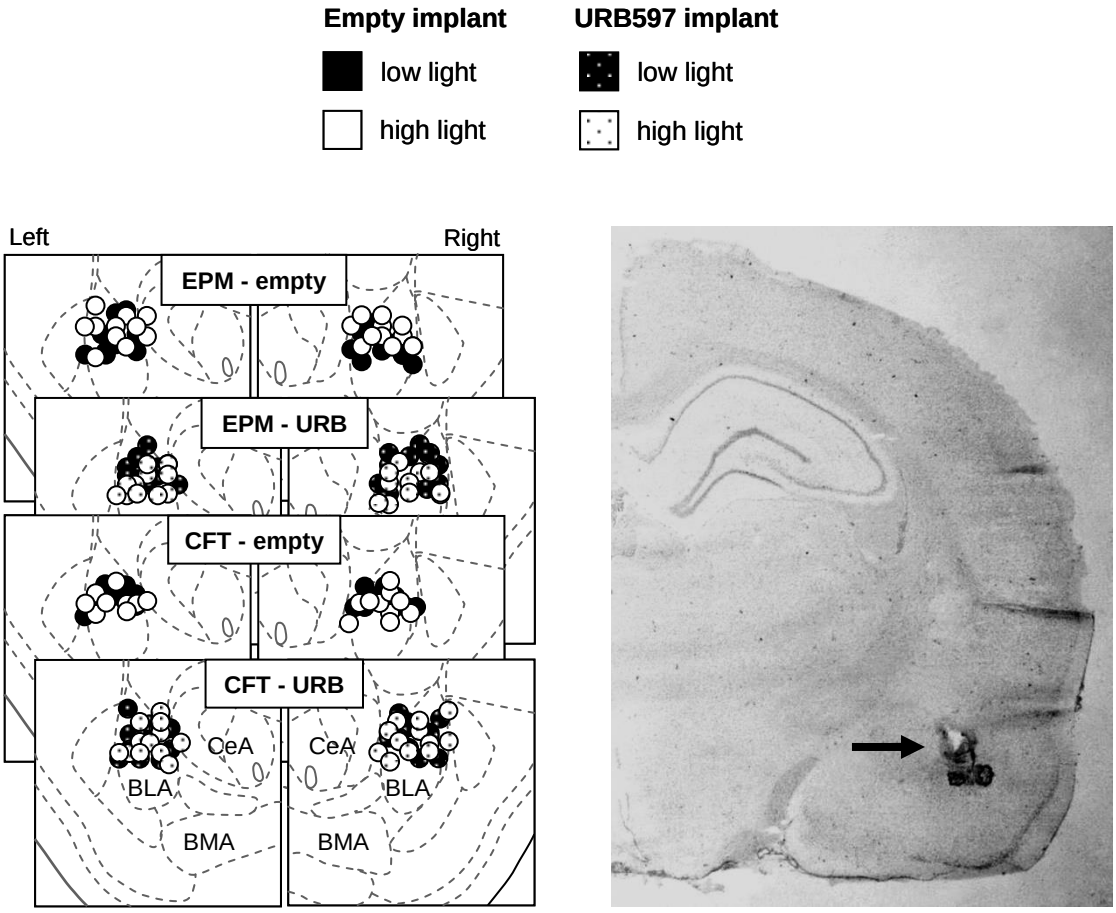
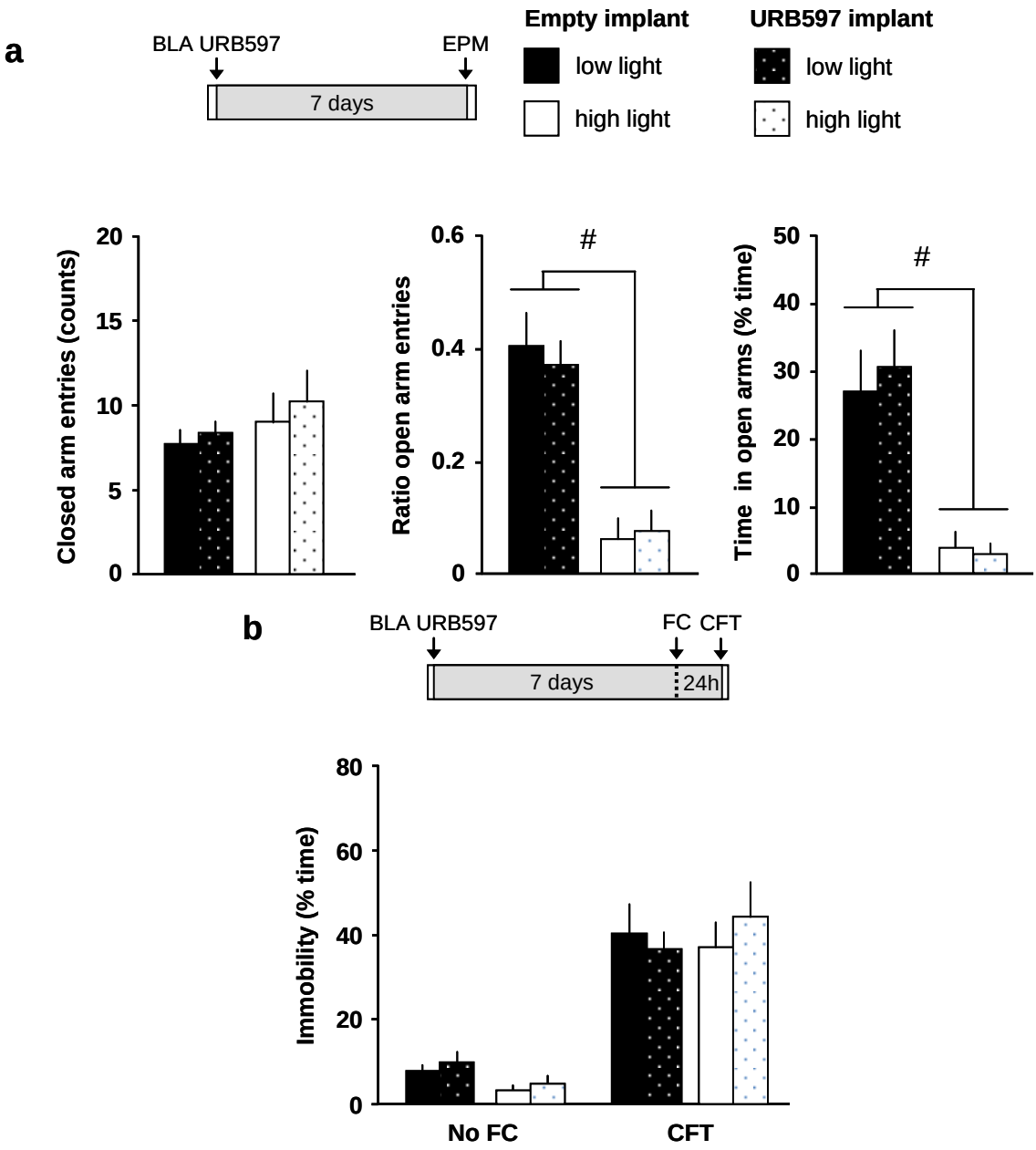

Empty implant
low light
high light
URB597 implant
low light
high light
Left
Right
EPM - empty
EPM - URB
CFT - empty
CFT - URB
CeA
CeA
BLA
BLA
BMA
BMA

Figure 5.


a
BLA URB597
EPM
7 days
Empty implant
low light
high light
URB597 implant
low light
high light
Closed arm entries (counts)
20
15
10
5
0
Ratio open arm entries
0.6
0.4
0.2
0
#
Time in open arms (% time)
50
40
30
20
10
0
#
b
BLA URB597
FC
CFT
7 days
24h
Immobility (% time)
80
60
40
20
0
No FC
CFT