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# Preservation of striatal cannabinoid CB1 receptor function correlates with the anti-anxiety effects of FAAH inhibition

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***Running title:*** FAAH inhibition and CB1 receptor function

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***Abbreviations:*** ACSF: artificial cerebrospinal fuid; AEA: anadamide; CB1R: cannabinoid CB1 receptor; EPSC: excitatory postsynaptic current; EPM: elevated plus maze; FAAH: fatty acid amide hydrolase; HPA: hypothalamic-pituitary-adrenal; IPSC: inhibitory postsynaptic current; OFT: open-field test; SDS: social defeat stress

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## Abstract

The endocannabinoid anandamide (AEA) plays a crucial role in emotional control, and inhibition of its degradation by the fatty acid amide hydrolase (FAAH) has a potent anti-anxiety effect. The mechanism by which magnification of AEA activity reduces anxiety is still largely undetermined.

By using FAAH mutant mice and both intraperitoneal and intracerebroventricular administration of the FAAH inhibitor URB597, we found that enhanced AEA signaling reversed, *via* central cannabinoid CB1 receptors (CB1Rs), the anxious phenotype of mice exposed to social defeat stress. This behavioral effect was associated with preserved activity of CB1Rs regulating GABA transmission in the striatum, whereas these receptors were dramatically down-regulated by stress in control animals. The hypothalamic-pituitary-adrenal (HPA) axis was not involved in the anti-stress effects of FAAH inhibition, although HPA axis is a biological target of endogenous AEA. We also provided some physiological indications that striatal CB1Rs regulating GABA synapses are not the receptor targets of FAAH inhibition, which rather resulted in the stimulation of striatal CB1Rs regulating glutamate transmission. Collectively, our findings suggest that preservation of cannabinoid CB1 receptor function within the striatum is a possible synaptic correlate of the anti-anxiety effects of FAAH inhibition.

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## Introduction

The life span of the endocannabinoid anandamide (AEA) is regulated by the fatty acid amide hydrolase (FAAH), that cleaves and increases tissue levels of AEA (Kathuria et al., 2003; McKinney and Cravatt, 2005; Maccarrone et al., 2008). Enhancement of AEA signaling through FAAH inhibition has recently emerged as an interesting novel route in the treatment of mood disorders (Kathuria et al., 2003; Gobbi et al., 2005; Patel and Hillard, 2006; Bortolato et al., 2007; Hill et al., 2007; Naidu et al., 2007; Cippitelli et al., 2008; Moreira et al., 2008; Rubino et al., 2008; Scherma et al., 2008; Haller et al., 2009; Micale et al., 2009). The mechanism by which FAAH inhibition results in ameliorated emotional control is largely undetermined, although magnification of AEA signaling at cannabinoid CB1 receptors (CB1Rs) has been implicated based on the fact that blockade of these receptors prevents the anxiolytic properties of both pharmacological and genetic inactivation of FAAH (Moreira et al., 2008; Haller et al., 2009; Micale et al., 2009). CB1Rs are particularly abundant in the striatum, a subcortical brain area recognized to play an important role in anxiety-related behaviors (Rogan et al., 2005; Favilla et al., 2008), and involved in the AEA-elevating effects of mood enhancing doses of URB597, a potent and selective FAAH inhibitor (Bortolato et al., 2007). As in other brain areas, striatal CB1Rs are heterogeneously distributed in presynaptic neuronal elements, and regulate both glutamate and GABA release resulting in contrasting effects on striatal neuron activity and output (Szabo et al., 1998; Huang et al., 2001; Gerdeman and Lovinger, 2001; Andersson et al., 2005; Ade and Lovinger, 2007).

We have recently reported that the anxious phenotype of mice exposed to social defeat stress is associated with loss of sensitivity of CB1Rs controlling GABA synapses (CB1Rs$_{(GABA)}$) in the striatum (Rossi et al., 2008), and that potentially rewarding experiences contrast the behavioral consequences of stress by enhancing CB1R$_{(GABA)}$ function (De Chiara et al., 2010). In contrast, CB1Rs regulating striatal glutamate transmission (CB1Rs$_{(Glu)}$) are unaffected by both stress and rewards (Rossi et al., 2008; De Chiara et al., 2010). These findings selectively implicate striatal CB1Rs$_{(GABA)}$ in emotional control, and suggest that FAAH inhibition exerts anti-anxiety and

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antidepressant effects by activating these receptors. On the other hand, however, systemic URB597 administration decreases plasma corticosterone levels in stressed mice (Patel et al., 2004), and the inhibition of hypothalamic-pituitary-adrenal (HPA) axis might well explain the effects of FAAH inhibition against stress. In fact, pharmacological inhibition of FAAH activity within the basolateral amygdala complex has been shown to attenuate stress-induced corticosterone secretion (Hill et al., 2009). Moreover, blockade of glucocorticoid receptors with RU486 prevents stress-induced striatal $CB1R_{(GABA)}$ inactivation, while intraperitoneal (i.p.) corticosterone blocks these stress-sensitive receptors (Rossi et al., 2008).

The relationship between stress and the endocannabinoid system is complex, likely depending on the type of stressful event and the brain area considered. In fact, the activation of CB1Rs mediates different behavioral responses to stress, such as stress-induced analgesia (Hohmann et al., 2005), stress-induced inhibition of reproductive behaviors (Coddington et al., 2007), and stress-induced increased emotionality (Hill and Gorzalka, 2006). It also reduces stress-induced depressive symptoms (Gobbi et al., 2005; Rademacher and Hillard, 2007), inappropriate retention of aversive memories (Ganon-Elazar and Akirav, 2009), and cognitive deficits (Hill et al., 2005).

The present study was specifically designed to clarify the mechanisms by which FAAH inhibition protects from stress-induced effects in the striatum.

## Materials and Methods

Male C57/bl6 mice (8-10 weeks old) were used for all the experiments. All animals were housed, four per cage, on a 12 h light/dark cycle with lights on at 06:00 h. All efforts were made to minimize animal suffering and to reduce the number of mice used, in accordance with the European Communities Council Directive of 24 November, 1986 (86/609/EEC).

### *Psychoemotional stress*

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Social defeat stress (SDS) was induced as already described (Avgustinovich et al., 2005; Berton et al., 2006; Rossi et al., 2008; De Chiara et al., 2010). Briefly, individual mice were subjected to daily bouts for 10 min direct sensory contact with an aggressive CD1 resident mouse, followed by 3 hours protected sensory contact with their aggressor. Mice were exposed to a different aggressor each day for 3 days.

### *Behavior*

To measure anxiety, the Open Field Test (OFT) and the Elevated Plus Maze (EPM) paradigm were used. The two tests were administered two hours apart in random sequence in each group of animals and in their appropriate controls (8 mice per group). Stressed mice underwent the behavioral analysis 24 hours after the last session of aggression.

The OFT paradigm assesses motor activity of animals in an averse, stressful environment. This protocol was performed as previously reported (Errico et al., 2008; De Chiara et al., 2010). Briefly, mice were placed into the center of a clear Plexiglas arena (25 x 35 x 20 cm) in which they were allowed to explore for 30 min. Overhead incandescent light bulbs provided a 600 lux illumination inside the test chamber. Total and center distance were recorded using a video tracking system (Videotrack, Viewpoint S.A., Champagne au Mont d’Or, France). Center distance was divided by the total distance to obtain a centre/total distance ratio used as an index of anxiety-related behavior.

The EPM represents one of the most widely used tests for assessing anxiety in rodents (Lister, 1987). Each mouse was placed in the center of the maze with its nose in a closed arm. The time spent in the open arms and in the closed arms of the maze was recorded as measure of anxious state. The time spent in each compartment was expressed as percentage of the total 5 minutes test time. The entry with all four feet into one arm was defined as an arm entry.

At the end of each trial the arena of the OFT and the maze were wiped clean.

### *Electrophysiology*

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Whole-cell patch clamp recordings from single striatal neurons in corticostriatal coronal slices (200 µm) were performed as previously described (Rossi et al., 2008; De Chiara et al., 2010). To detect spontaneous (sIPSCs) and miniature GABAA-mediated inhibitory postsynaptic currents (mIPSCs), intraelectrode solution had the following composition (mM): CsCl (110), $K^+$-gluconate (30), ethylene glycol-bis (ß-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA; 1.1), HEPES (10), $CaCl_2$ (0.1), Mg-ATP (4), Na-GTP (0.3). MK-801 (30 µM) and CNQX (10 µM) were added to the external solution to block, respectively, NMDA and nonNMDA glutamate receptors. Conversely, to study spontaneous glutamate-mediated excitatory postsynaptic currents (sEPSCs), the recording pipettes were filled with internal solution of the following composition: (mM) $K^+$-gluconate (125), NaCl (10), $CaCl_2$, (1.0), $MgCl_2$ (2.0), 1,2-bis (2-aminophenoxy) ethane-N,N,N,N-tetraacetic acid (BAPTA; 0.5), N-(2-hydroxyethyl)-piperazine-N-s-ethanesulfonic acid (HEPES; 19), guanosine triphosphate (GTP; 0.3), Mg-adenosine triphosphate (Mg-ATP; 1.0), adjusted to pH 7.3 with KOH. Bicuculline (10 µM) was added to the perfusing solution to block GABAA-mediated transmission.

The detection threshold of sIPSCs, mIPSCs or sEPSCs was set at twice the baseline noise. The fact that no false events would be identified was confirmed by visual inspection for each experiment. Offline analysis was performed on spontaneous and miniature synaptic events recorded during fixed time epochs (5-10 samplings of 2-3 min duration each, recorded every 2-3 minutes), for a total of 10 to 30 min analysis for each recorded neuron, depending on the length of the experiment.

To study the effects of stress on both striatal $CB1Rs_{(GABA)}$ and $CB1Rs_{(Glu)}$, mice were killed and corticostriatal brain slices prepared 24 after the last session of aggression.

### *Drugs*

Corticosterone (from Sigma-RBI, St. Louis, USA) was administrated subcutaneously (s.c.) once a day in a volume of 10 ml/kg for 3 consecutive days (20 mg/kg, suspended in physiological saline containing 0.1% DMSO and 0.1% Tween-80). In other experiments, a single dose of URB597 (0,3 mg/kg, dissolved in DMSO, from Alexis Biochemicals, San Diego, CA, USA), alone or in combination with AM251 (6 mg/kg, dissolved in in saline with DMSO 10% and Tween 80.5%, from Tocris, Bristol, UK) was injected intraperitoneal (i.p.) in non stressed animals or immediately after the third of three sessions of stress. The injected volume was similar in single and combined treatments. In other experiments URB597 (1µM dissolved in 1µl DMSO) was also administered *in vivo* by a intracerebroventicular (i.c.v.) injection under stereotaxic coordinates (A. +0; L. +0.8 and D. -2.4) and general anaesthesia with 2,2,2-tribromoethanol (10 mg/ml; 1/27 of body weight) at the end of the three sessions of stress. Mice receiving s.c., i.p., or i.c.v. injections of the appropriate volume of vehicle were used as controls.

Drugs used for the electrophysiological experiments were first dissolved in DMSO (AM251, HU210, URB597) or water, then in the bathing ACSF to the desired final concentration. The concentrations of the various drugs were chosen according to previous in vitro studies on corticostriatal brain slices (Rossi et al., 2008; Maccarrone et al., 2008), and were as follows: CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione, 10 µM), AM251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide, 10 µM), HU210 ((6aR)-trans-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol 1 µM), MK-801 ((5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cycl ohepten-5,10-imine maleate, 30 µM), tetrodotoxin (TTX, Octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol, 1 µM) (from Tocris, Bristol, UK). Bicuculline ((6*R*)-6-[(5*S*)-6-methyl-5,6,7,8-tetrahydro[1,3]dioxolo[4,5-*g*]isoquinolin-5-yl]furo[3,4-*e*][1,3]benzodioxol-8(6*H*)-one, 10 µM) (from Sigma-RBI, St. Louis, USA). URB597 ([3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate, 1 µM) (from Alexis Biochemicals, San Diego, CA, USA). In the experiments with drugs dissolved in DMSO the control samplings were obtained during DMSO and ACSF applications.

### *Statistical analysis*

The analyses were performed on a per-animal basis, and throughout the text "n" refers to the number of mice used. Eight mice were used for each behavioral experiment, and 5 to 8 mice were employed for a single electrophysiological experiment. Electrophysiological results from neurons recorded from the same animal were treated as a separate sample and averaged prior to calculating statistics. One to 6 neurons per animal were recorded. For data presented as the mean ± S.E.M., statistical analysis between two groups was performed using a paired or unpaired Student's t-test or Wilcoxon's test. Multiple comparisons were analysed by one-way ANOVA followed by Tukey HSD. One or two animals per day were used for the electrophysiology. The significance level of the results was established at $p<0.05$.

## Results

### *Systemic URB597 protects from SDS-induced anxiety*

The anti-anxiety properties of i.p. URB597 have been tested in experimental conditions not including SDS (Kathuria et al., 2003; Patel and Hillard, 2006; Scherma et al., 2008; Moreira et al., 2008; Haller et al., 2009; Micale et al., 2009). Thus, mice were injected with a single i.p. dose of URB597 or of vehicle at the end of the SDS protocol, and the effects on both OFT and EPM were evaluated 24 hours later. At the OFT, the activity in the center of arena and the center/total distance ratio can be used as an index of anxiety-related responses, as anxiety reduces the time spent in the center of the arena. ANOVA analysis showed a significant increase in anxiety in mice exposed to stress, as they spent a shorter time in the center of the arena compared with standard-housed mice (center time: $F=14.35$, $p<0.001$; center entry count: $F=22.95$, $p<0.0001$; center/total distance ratio: $F= 20.44$, $p<0.0001$) (n=8 for each groups). URB597 significantly reduced the anxious phenotype of stressed mice only receiving vehicle (post hoc comparison versus stressed untreated mice: center time $p< 0.05$, center entry count $p<0.05$, center/total distance ratio $p<0.01$) (Fig. 1A).

Reduced anxiety in mice exposed to URB597 was also seen at the EPM. All EPM measures were significantly modified by the SDS paradigm (percentage of time spent in open arms: $F=14.77$,

$p<0.001$ percentage of time spent in closed arms: F=9.3, $p=0.001$) (n=8 for each groups). In contrast, stressed animals exposed to URB597 showed a significant increase in the time spent in the open arms ($p<0.05$) and a reduction in the time spent in the closed arms ($p<0.05$) (Fig. 1B).

### *Systemic FAAH inhibition protects from SDS-induced CB1R$_{(GABA)}$ blockade*

Consistent with the behavioral data, we also found that a single i.p. administration of URB597 was able to rescue the function of stress-sensitive striatal CB1Rs$_{(GABA)}$. According to previous experiments, in fact, the CB1R agonist HU210 reduced GABA-mediated sIPSC frequency in control mice (n=5, $p<0.01$), while it failed to affect GABA transmission following SDS (n=7, $p>0.05$) (Rossi et al., 2008; De Chiara et al., 2010) (Fig. 2A). HU210, however, inhibited the frequency of sIPSCs in stressed mice treated with i.p. URB597 (n=8, $p<0.01$), and it was ineffective in stressed mice only receiving the vehicle (n=6, $p>0.05$) (Fig. 2B). Of note, i.p. URB597 (n=5) failed to alter HU210-mediated sIPSC inhibition in non-stressed mice ($p<0.05$ compared to pre-HU210 values, and $p>0.05$ compared to HU210 effects in mice receiving i.p. vehicle (n=5)) (Fig. 2C).

FAAH -/- mice (Cravatt et al., 2001; Maccarrone et al., 2008) display reduced anxious behavior (Moreira et al., 2008), and thus we tested whether the activity of stress-sensitive CB1Rs$_{(GABA)}$ was preserved after SDS in these mutants, as seen in response to i.p. URB597. In non-stressed FAAH -/- mice, HU210-induced inhibition of sIPSC frequency was similar ($p>0.05$) to that observed in the respective wild-type (WT) counterparts (n=5, $p<0.01$ for the two groups compared to pre-HU210 values). Following SDS, however, HU210 was still able to reduce striatal sIPSCs in FAAH-/- mice (n=5, $p<0.01$), while it was ineffective in control animals (n=5, $p>0.05$) (Fig. 2D,E).

### *Inhibition of FAAH activity preferentially modulates CB1Rs$_{(Glu)}$*

To uncover the preferential synaptic target of endogenous AEA in the striatum, we recorded both GABA-mediated sIPSCs and glutamate-mediated sEPSCs, before and during the application of

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URB597. URB597, which significantly increases AEA but not 2-arachidonoylglicerol (2-AG) levels in striatal slices (Maccarrone et al., 2008), failed to affect sIPSC frequency (n=6, $p>0.05$, Fig. 3A) and amplitude (n=6, 98±3.1% respect to pre-drug, $p>0.05$, not shown), while it significantly reduced sEPSC frequency (n=6, $p<0.01$, Fig. 3B). sEPSC amplitude was conversely unaltered by URB597 (n=6, $p>0.05$, 101±2.8% respect to pre-drug, $p>0.05$, not shown). Inhibition of CB1Rs with the selective antagonist AM251 failed to alter *per se* sEPSC frequency (n=6, $p>0.05$, 97±3.3% respect to pre-drug, $p>0.05$, not shown), but fully prevented the action of URB597 on sEPSC frequency (n=6, $p>0.05$), indicating that endogenous AEA preferentially activates presynaptic CB1Rs$_{(Glu)}$ following FAAH inhibition (Maccarrone et al., 2008; Musella et al., 2009) (Fig. 3B).

Consistent results were obtained in brain slices from FAAH-/- mice. In these animals, we assumed that tonic activation of CB1Rs$_{(GABA)}$ or of CB1Rs$_{(Glu)}$ by elevated AEA content (Maccarrone et al., 2008) could be uncovered by measuring the effects of CB1R blockade. AM251 failed to increase striatal sIPSC frequency in FAAH-/- mice (n=5) and in their respective control group (n=6) ($p>0.05$ compared to pre-drug values for both experimental groups). We therefore analyzed the effects of AM251 on CB1Rs$_{(Glu)}$ in control and in FAAH-/- mice. AM251 failed to alter the frequency of sEPSCs in WT mice (n=6 and $p>0.05$ respect to pre-drug values), while it significantly increased sEPSC frequency in FAAH-/- mice (n=5 and $p<0.01$ respect to pre-drug values) (Fig. 3C).

As expected for the presence of a tonic inhibition of glutamate release in FAAH-/- mice (Musella et al., 2009), the basal frequency (WT: 2.7 ± 0.3 Hz, FAAH-/-: 1.6 ± 0.4 Hz; n=11 for both groups; $p<0.05$) but not the amplitude (WT: 13.5 ± 1.6 pA, FAAH-/-: 14.5 ± 1.8 pA; n=11 for both groups; $p>0.05$) of sEPSCs was significantly lower in these mutants (not shown).

### *FAAH inhibition protects from stress independently of glucocorticoid activity*

FAAH inhibition reduces plasma corticosterone concentrations following stress (Patel et al., 2004), implying that preserved activity of CB1Rs$_{(GABA)}$ in FAAH -/- mice exposed to SDS might be a consequence of reduced glucocorticoid activity in the brain. Accordingly, systemic corticosterone

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administration results in $\text{CB1R}_{\text{(GABA)}}$ blockade in non-stressed animals (Rossi et al., 2008). Thus, we administered corticosterone in FAAH-/- mice, to see whether this treatment was able to block striatal $\text{CB1Rs}_{\text{(GABA)}}$ in these mice. Corticosterone treatment fully prevented HU210-induced inhibition of sIPSC frequency in control mice (n=6, p>0.05), while it was ineffective in FAAH-/- mice (n=6, p<0.01). These results indicate that reduced glucocorticoid activity is not involved in the effects of FAAH inhibition on striatal $\text{CB1Rs}_{\text{(GABA)}}$ following stress (Fig. 4A).

### *Selective inhibition of FAAH in the brain protects $\text{CB1Rs}_{\text{(GABA)}}$ from stress*

Exogenous corticosterone was unable to overcome the protective effects of FAAH-/- mice on the synaptic consequences of stress, indicating that preservation of $\text{CB1R}_{\text{(GABA)}}$ activity in these mice does not rely on the inhibition of the HPA axis by FAAH inhibition. Rather, these findings suggest that FAAH inhibition protects $\text{CB1Rs}_{\text{(GABA)}}$ from the effects of both stress and corticosterone by acting centrally.

Thus, we measured HU210 effects in mice receiving URB597 through a single i.c.v. injection at the end of the SDS protocol. As with i.p. URB597, HU210 inhibited sIPSC frequency in SDS mice treated with i.c.v. URB597 (n=6, p<0.01), but not with i.c.v. vehicle (n=6, p>0.05) (Fig. 4B).

### *CB1Rs mediate the emotional effects of FAAH blockade*

We then addressed the hypothesis that CB1Rs mediate the anti-anxiety effects of URB597. Blockade of CB1R with AM251 prevented the anxiolytic properties of URB597. In fact, combined i.p. URB597 plus AM251 administration after SDS failed to alter the behavioral measures recorded at both OFT (post hoc comparisons versus stressed untreated mice: center time p>0.05, center entry count p>0.05, center/total distance ratio p>0.05; n=8 for each groups) and EPM (post hoc comparisons versus stressed untreated mice: percentage of time in open arms p>0.05, percentage of time in closed arms p>0.05; n=8 for each groups) (Fig. 5A,B). AM251 also blocked URB597 effects on striatal sIPSCs recorded from stressed animals. In fact, URB597 plus AM251 failed to preserve the HU210-induced inhibition of sIPSC frequency (n=5, p>0.05 respect to pre-HU210 values) (Fig. 5C).

## Discussion

A recent study identified FAAH as a critical molecule involved in mood control in humans, showing that carriers of a FAAH gene mutation with reduced enzyme activity had both decreased threat-related brain reactivity and reduced anxiety (Hariri et al., 2009). These findings are particularly relevant because they allow generalizing to humans the results of the existing literature on the anti-anxiety effects of reduced FAAH activity in rodents. Both genetic and pharmacological inactivation of FAAH, in fact, exerts anxiolytic and antidepressant actions in rodents (Kathuria et al., 2003; Gobbi et al., 2005; Patel and Hillard, 2006; Bortolato et al., 2007; Hill et al., 2007; Naidu et al., 2007; Cippitelli et al., 2008; Moreira et al., 2008; Rubino et al., 2008; Scherma et al., 2008; Haller et al., 2009; Micale et al., 2009), and does not cause sedation, hypothermia, hyperphagia or abuse potential (Fegley et al., 2005; Gobbi et al., 2005; Lichtman and Martin, 2005), which are important side effects of the direct CB1R agonist $\Delta^9$-tetrahydrocannabinol.

The results of the present study confirm and extend previous work showing that FAAH inhibition has a potent anti-anxiety activity. In addition, our investigation identifies a possible synaptic correlate of this activity in the protection of striatal CB1Rs controlling GABA synapses against the consequences of aversive experiences. FAAH blockade did not induce *per se* sensitization of striatal CB1Rs$_{(GABA),}$ since HU210 effects on sIPSC frequency were not potentiated by both pharmacological and genetic inhibition of FAAH in non-stressed mice. In contrast, the receptor enhancing effects of FAAH blockade was only seen in conditions causing functional downregulation of striatal CB1Rs$_{(GABA)}$, such as after the SDS protocol or after systemic corticosterone administration. The lack of effect of FAAH inhibition on basal CB1R$_{(GABA)}$ function might contribute to explain why the anxiolytic properties of both URB597 (Naidu et al., 2007;

Haller et al., 2009) and FAAH genetic knockout (Naidu et al., 2007) are less evident in non-stressed animals.

We identified CB1Rs controlling glutamate transmission as plausible receptor targets of FAAH inhibition in the striatum, suggesting that the interaction between striatal CB1Rs$_{(Glu)}$ (activated by AEA following FAAH inhibition) and CB1Rs$_{(GABA)}$ (preserved from stress-induced down-regulation following FAAH inhibition) might play a role in mood control. This interaction might have other physiological roles than the control of social stress effects, since we have observed that activation of striatal CB1Rs$_{(Glu)}$ by endogenous AEA is also able to preserve the functional integrity of CB1Rs$_{(GABA)}$ after systemic administration of corticosterone, which likely regulates synaptic activity in multiple physiological and pathological conditions, including systemic inflammatory (Beishuizen et al., 2003; Bornstein et al., 2008) and metabolic states (Macfarlane et al., 2008), and circadian rhythms (Seckl and Meaney, 2004; Cutolo et al., 2006), all associated with significant changes of glucocorticoid plasma levels.

The data showing that stress-induced anxiety is associated with loss of striatal CB1R$_{(GABA)}$ activity (Rossi et al., 2008), and that the anti-anxiety effects of natural rewards (De Chiara et al., 2010) or of FAAH inhibition (present work) are paralleled by the recovery of these receptors are in line with previous findings emphasizing the involvement of striatal neuron activity in the control of anxiety-related behavior in humans (Reiman et al., 1989; Yoo et al., 2005; Mathew and Ho, 2006), and in rodents (Favilla et al., 2008).

CB1Rs reduce transmitter release by inhibiting calcium channels and cAMP levels in presynaptic nerve terminals (Piomelli et al., 2003; Howlett et al., 2004), suggesting that the loss of CB1R sensitivity paralleling the anxious behavior in stressed mice enhances cAMP signaling in striatal neurons. Importantly, enhancement of cAMP signaling in the striatum through genetic deletion of the cAMP-degrading enzyme phosphodiesterase 4B (Zhang et al., 2008), overexpression of the striatally enriched cAMP-generating enzyme adenylyl cyclase 5 (Kim et al., 2008), or chronic expression in the striatum only of a constitutively active G-protein stimulating adenylyl cyclase activity (Favilla et al., 2008) are all associated with increased anxiety in mice, confirming the relevance of our findings for the pathophysiology of anxiety, and for its treatment. In this respect, it is also noteworthy that pharmacological potentiation of adenylyl cyclase-cAMP activity has been found to selectively increase GABA release in brain areas also including the striatum (Hack et al., 2003; Murphy et al., 2003; Harvey and Stephens, 2004; Centonze et al., 2008), suggesting that manipulations enhancing both striatal cAMP levels and anxious behavior (Favilla et al., 2008; Kim et al., 2008; Zhang et al., 2008) result in increased inhibition of striatal neuron activity, possibly disrupting a circuitry normally limiting fearful or anxiety-related behaviors (Rogan et al., 2005). In line with this hypothesis, and consistent with the findings of the present study, reduced FAAH activity has been associated with both increased striatal activation and reduced anxiety in humans (Hariri et al., 2009).

Along with the effects on $CB1R_{(GABA)}$ function in the striatum here reported, the anti-anxiety effects of systemic FAAH inhibition likely involve other actions in different stress-sensitive brain areas. Pharmacological inhibition of FAAH activity within the basolateral amygdala, or direct pharmacological activation of CB1Rs in this brain area, in fact, significantly reduce stress-induced corticosterone secretion (Herman et al., 2003; Hill et al., 2009; Ganon-Elazar and Akirav, 2009). Only direct pharmacological inhibition of FAAH activity within the striatum might therefore allow clarifying how the synaptic effects described in the present work contribute to the behavioral effects of URB597 against stress.

Understanding the synaptic underpinning of emotional control is essential for the development of effective strategies against neuropsychiatric conditions such as anxiety, phobias, obsessive-compulsive disorder, and depression.

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## Footnotes

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## Figure Legends

**Figure 1.** FAAH inhibition protects from SDS-induced anxiety. A. The graphs show behavioral measures recorded at the OFT. I.p. administration of URB597 increased the activities in the center of arena (time spent in the center, count of the entries into the center) and the center/total distance ratio after SDS. B. The graphs show behavioral measures recorded at the EPM. I.p. URB597 after SDS increased the time spent in the open arms and reduced the time spent in the closed arms.

* $p<0.05$, ** $p<0.01$ versus control

# $p<0.05$. ## $p<0.01$ versus SDS

**Figure 2.** FAAH inhibition protects from SDS-induced $CB1R_{(GABA)}$ blockade. A. Stimulation of CB1Rs with HU210 reduced sIPSC frequency in control mice. This effect was fully abolished in neurons from mice exposed to SDS. B. I.p. URB597 was able to rescue the effect of HU210 on sIPSC frequency in mice exposed to SDS. C. I.p. URB597 failed to alter *per se* HU210-mediated sIPSC inhibition in control mice. D. HU210 was able to reduce striatal sIPSCs in FAAH-/- mice exposed to SDS, while it was ineffective in stressed WT animals. Examples of voltage-clamp recordings of sIPSCs before and during the application of HU210 in WT and FAAH-/- exposed to SDS are shown on the right. E. In FAAH-/- mice, HU210-induced inhibition of sIPSC frequency was similar to that observed in WT mice.

* $p<0.05$ versus control

**Figure 3.** FAAH inhibition results in $CB1R_{(Glu)}$ but not $CB1R_{(GABA)}$ activation. A. Bath application of URB597 failed to affect sIPSC frequency. B. Bath application of URB597 inhibited sEPSC frequency. Blockade of CB1Rs with AM251 blocked the effects of URB597 on sEPSCs. C. AM251 did not increase striatal sIPSC frequency in WT and in FAAH-/- mice. Conversely, it significantly increased sEPSC frequency in FAAH-/- mice.

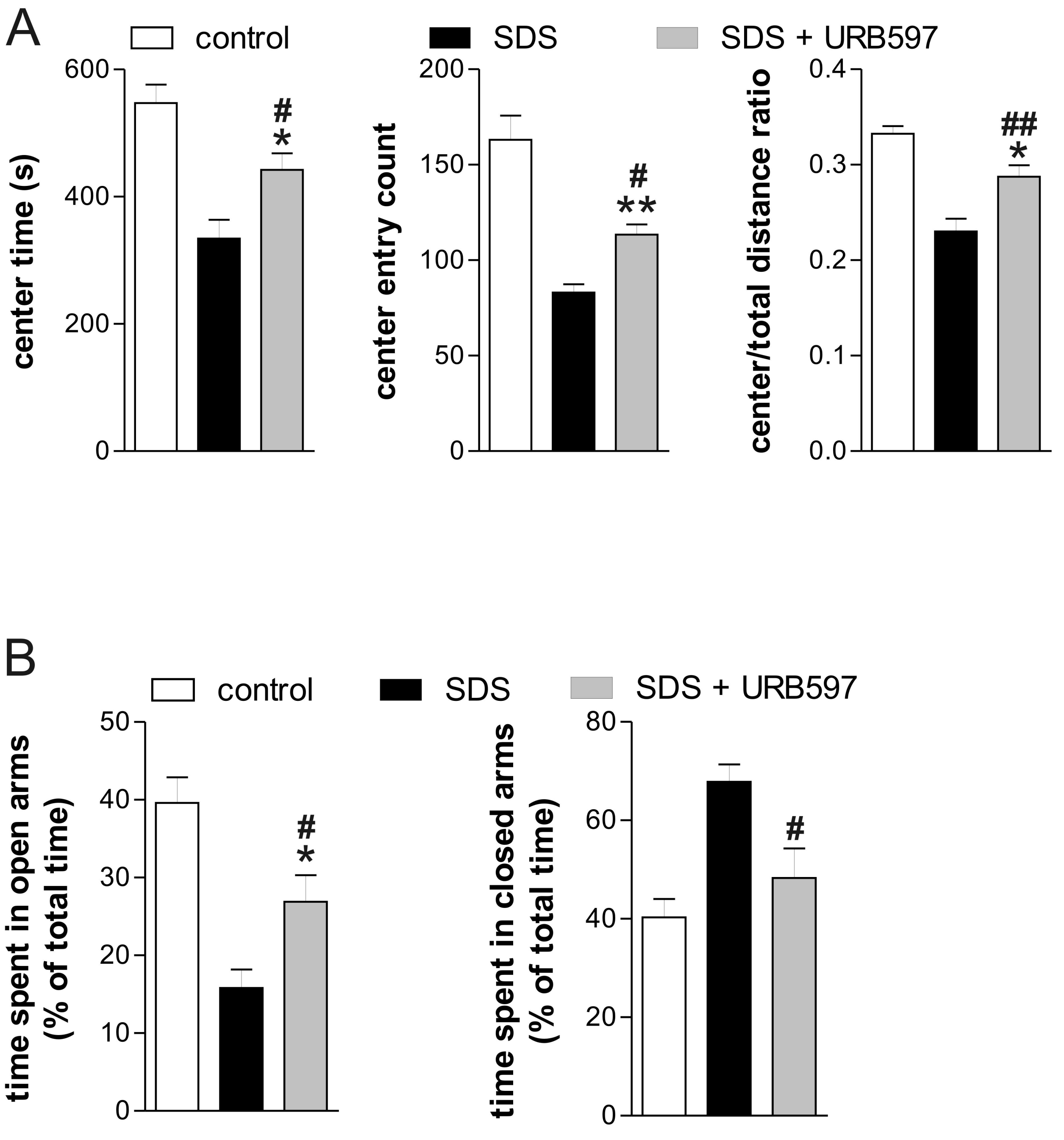
* $p<0.05$ versus control

**Figure 4.** FAAH inhibition protects from stress independently of glucocorticoid activity. A. Corticosterone treatment mimicked the stress effects on HU210-induced reduction of sIPSC frequency in WT mice, while it was ineffective in FAAH-/- mice. Examples of voltage-clamp recordings of sIPSC before and during the application of HU210 in WT and FAAH-/- mice treated with corticosterone are shown on the right. B. The graph shows that i.c.v. URB597 was able to rescue the effect of HU210 on sIPSC frequency after SDS.

**Figure 5.** CB1Rs are involved in the anti-anxiety effects of FAAH inhibition. A. The graphs show behavioral measures recorded at the OFT. Blockade of CB1Rs with AM251 fully prevented the anxiolytic properties of URB597 after SDS. B. Co-administration of URB597 and AM251 in stressed mice failed to increase the time spent in the open arms and to reduce the time spent in the closed arms at the EPM. C. URB597 plus AM251 failed to preserve the HU210-induced inhibition of sIPSC frequency.

* $p<0.05$, ** $p<0.01$ versus control

figure 1


A
control
SDS
SDS + URB597
center time (s)
600
400
200
0
#
*
center entry count
200
150
100
50
0
#
**
center/total distance ratio
0.4
0.3
0.2
0.1
0.0
##
*
B
control
SDS
SDS + URB597
time spent in open arms
(% of total time)
50
40
30
20
10
0
#
*
time spent in closed arms
(% of total time)
80
60
40
20
0
#

# figure 2

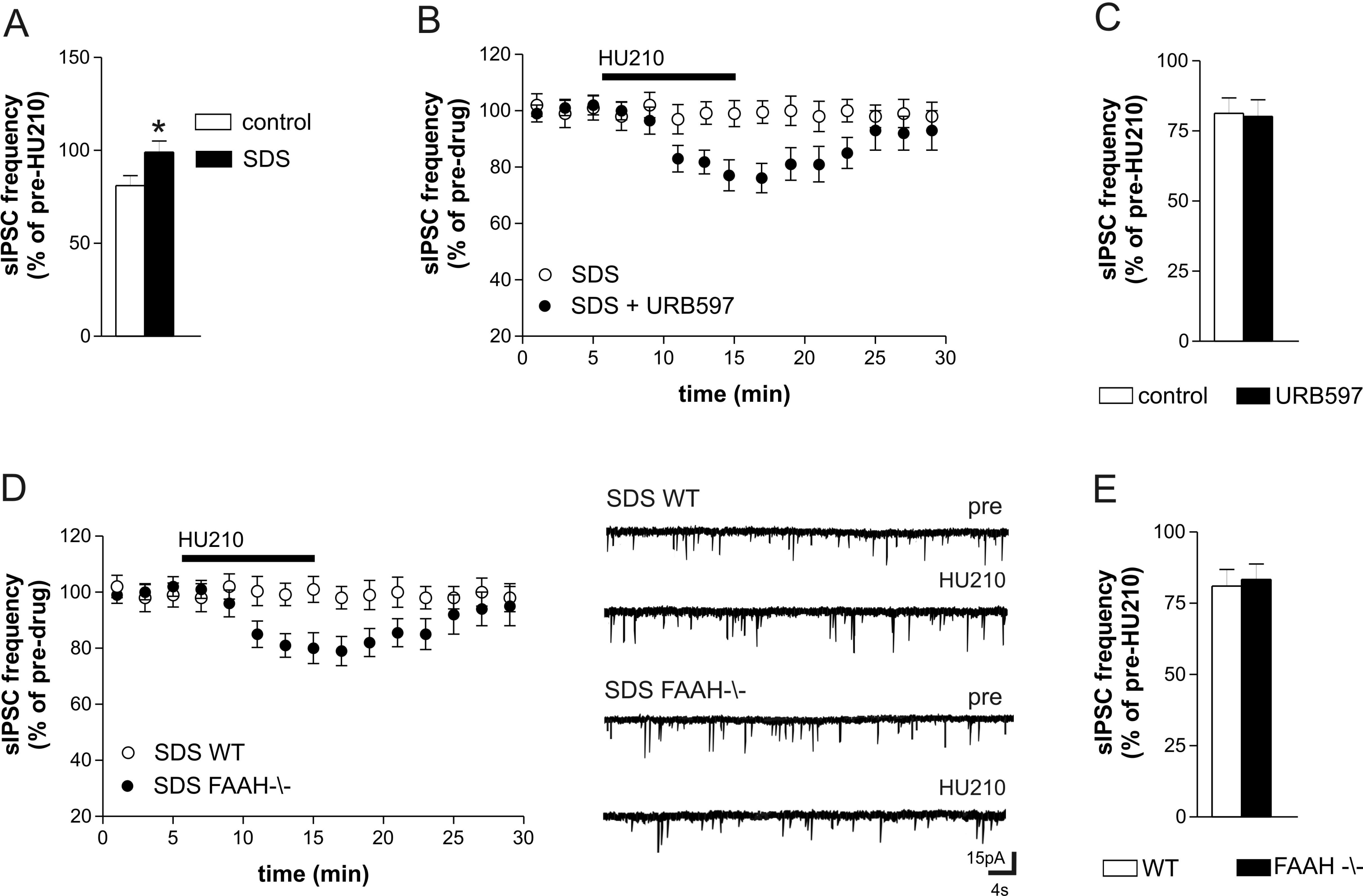
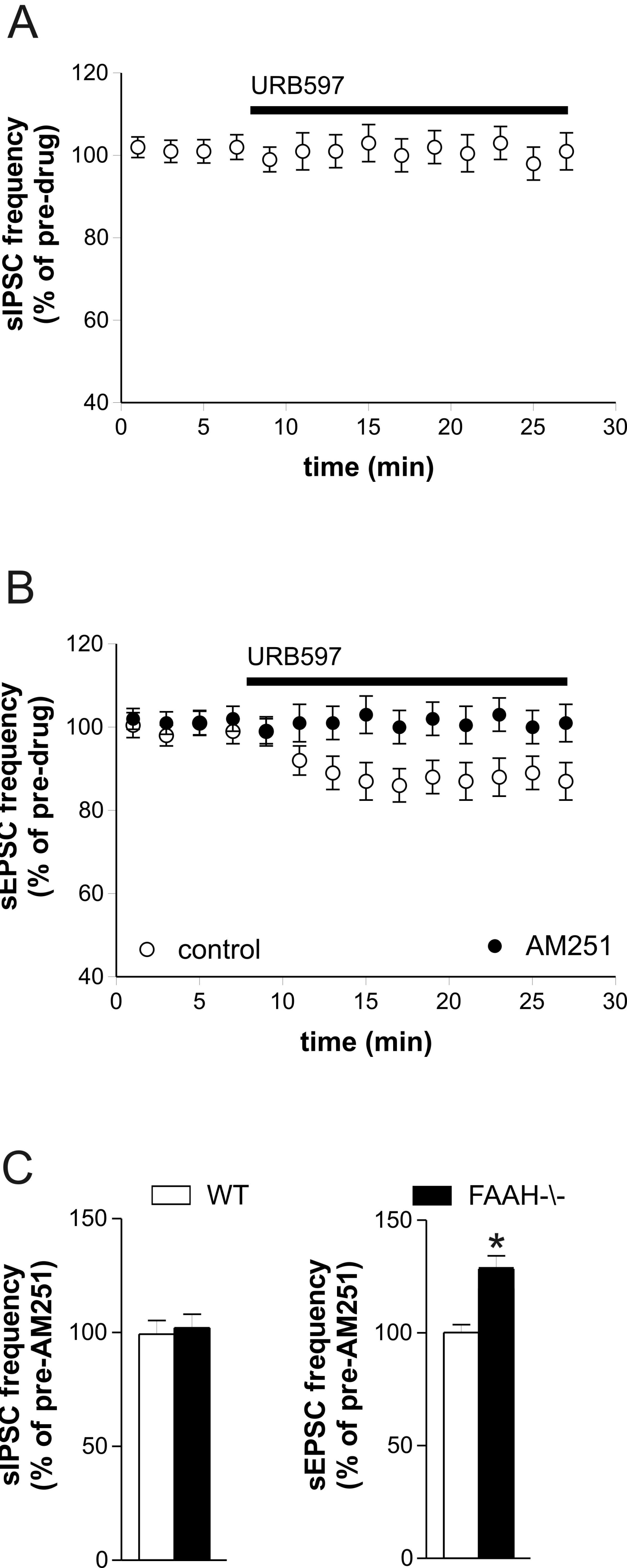

A
150
100
50
0
sIPSC frequency (% of pre-HU210)
*
control
SDS
B
HU210
120
100
80
60
40
20
sIPSC frequency (% of pre-drug)
SDS
SDS + URB597
0
5
10
15
20
25
30
time (min)
C
100
75
50
25
0
sIPSC frequency (% of pre-HU210)
control
URB597
D
HU210
120
100
80
60
40
20
sIPSC frequency (% of pre-drug)
SDS WT
SDS FAAH-\-
0
5
10
15
20
25
30
time (min)
SDS WT
pre
HU210
SDS FAAH-\-
pre
HU210
15pA
4s
E
100
75
50
25
0
sIPSC frequency (% of pre-HU210)
WT
FAAH -\-

figure 3


A
URB597
sIPSC frequency (% of pre-drug)
120
100
80
60
40
0
5
10
15
20
25
30
time (min)
B
URB597
sEPSC frequency (% of pre-drug)
120
100
80
60
40
○ control
● AM251
0
5
10
15
20
25
30
time (min)
C
WT
FAAH-\-
sIPSC frequency (% of pre-AM251)
150
100
50
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sEPSC frequency (% of pre-AM251)
150
100
50
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figure 4

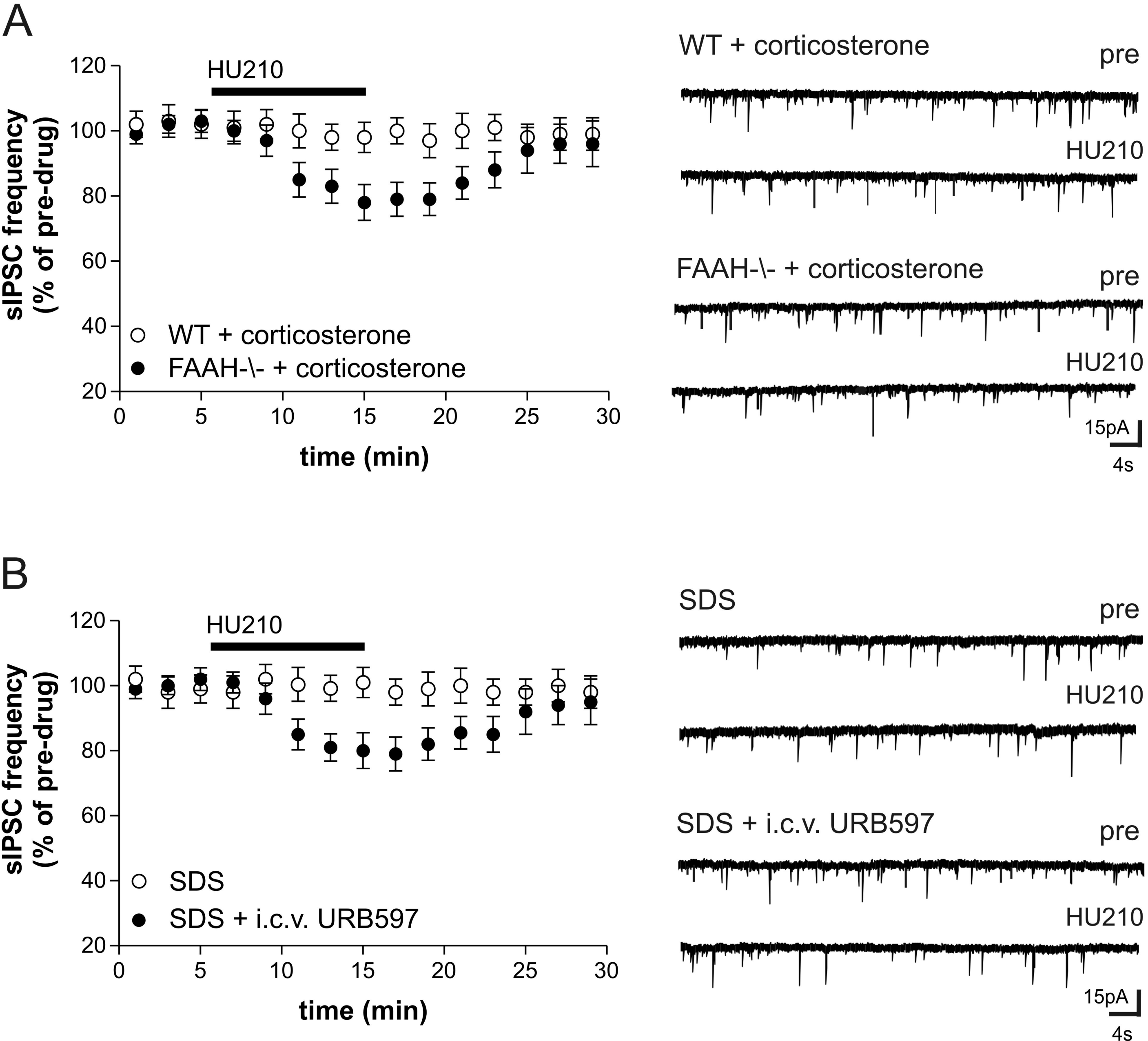
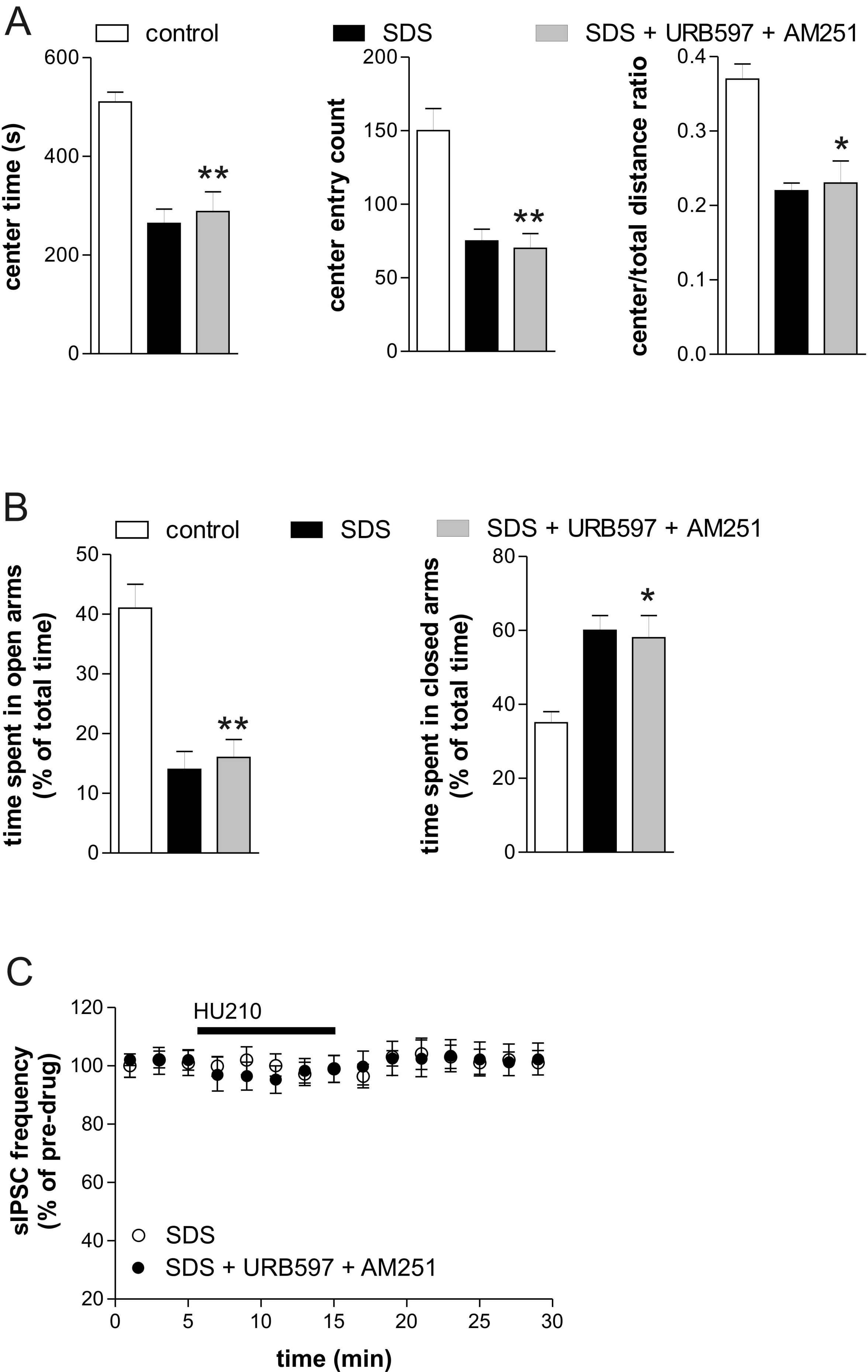

A
HU210
120
100
80
60
40
20
sIPSC frequency (% of pre-drug)
0
5
10
15
20
25
30
time (min)
○ WT + corticosterone
● FAAH-\- + corticosterone
WT + corticosterone
pre
HU210
FAAH-\- + corticosterone
pre
HU210
15pA
4s
B
HU210
120
100
80
60
40
20
sIPSC frequency (% of pre-drug)
0
5
10
15
20
25
30
time (min)
○ SDS
● SDS + i.c.v. URB597
SDS
pre
HU210
SDS + i.c.v. URB597
pre
HU210
15pA
4s

figure 5


A
control
SDS
SDS + URB597 + AM251
center time (s)
0
200
400
600
**
center entry count
0
50
100
150
200
**
center/total distance ratio
0.0
0.1
0.2
0.3
0.4
*
B
control
SDS
SDS + URB597 + AM251
time spent in open arms (% of total time)
0
10
20
30
40
50
**
time spent in closed arms (% of total time)
0
20
40
60
80
*
C
HU210
sIPSC frequency (% of pre-drug)
20
40
60
80
100
120
○ SDS
● SDS + URB597 + AM251
0
5
10
15
20
25
30
time (min)