

Preservation of Striatal Cannabinoid CB1 Receptor Function Correlates with the Antianxiety Effects of Fatty Acid Amide Hydrolase Inhibition

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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 antianxiety effect. The mechanism by which the 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 (3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate (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 antistress effects of FAAH inhibition, although the 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 antianxiety effects of FAAH inhibition.

The lifespan of the endocannabinoid anandamide (AEA) is regulated by the fatty acid amide hydrolase (FAAH), which 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 emerged recently 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

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ABBREVIATIONS: AEA, anandamide; CB1R, cannabinoid CB1 receptor; sEPSC, spontaneous excitatory postsynaptic current; EPM, elevated plus maze; FAAH, fatty acid amide hydrolase; sIPSC, spontaneous inhibitory postsynaptic current; OFT, open-field test; URB597, (3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate; HU210, (6a*R*)-*trans*-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b,d*]pyran-9-methanol; MK-801, (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate; DMSO, dimethyl sulfoxide; WT, wild type; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide; RU486, 17 β -hydroxy-11 β -[4-dimethylamino phenyl]-17 α -[1-propynyl]estra-4,9-dien-3-one; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid.

(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 are involved in the AEA-elevating effects of mood enhancing doses of (3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate (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; Gerdeman and Lovinger, 2001; Huang et al., 2001; Andersson et al., 2005; Ade and Lovinger, 2007).

We have reported recently 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 antianxiety and antidepressant effects by activating these receptors. On the other hand, systemic URB597 administration decreases plasma corticosterone levels in stressed mice (Patel et al., 2004), and the inhibition of hypothalamic-pituitary-adrenal 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 17 β -hydroxy-11 β -[4-dimethylamino phenyl]-17 α -[1-propynyl]estra-4,9-dien-3-one (RU486) prevents stress-induced striatal CB1R_(GABA) inactivation, whereas intraperitoneal corticosterone blocks these stress-sensitive receptors (Rossi et al., 2008).

The relationship between stress and the endocannabinoid system is complex, probably 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 6:00 AM. 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. Social defeat stress was induced as described previously (Avgustinovich et al., 2005; Berton et al., 2006; Rossi et al., 2008; De Chiara et al., 2010). In brief, individual mice

were subjected to daily bouts for 10 min of direct sensory contact with an aggressive CD1 resident mouse followed by 3-h 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) paradigms were used. The two tests were administered 2 h 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 h after the last session of aggression.

The OFT paradigm assesses the motor activity of animals in an aversive, stressful environment. This protocol was performed as reported previously (Errico et al., 2008; De Chiara et al., 2010). In brief, mice were placed into the center of a clear Plexiglas arena (25 \times 35 \times 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 center/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 a percentage of the total 5-min 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. Whole-cell patch-clamp recordings from single striatal neurons in corticostriatal coronal slices (200 μ m) were performed as described previously (Rossi et al., 2008; De Chiara et al., 2010). To detect spontaneous (sIPSCs) and miniature GABA_A-mediated inhibitory postsynaptic currents, intracellular solution had the following composition: 110 mM CsCl, 30 mM K⁺ gluconate, 1.1 mM EGTA, 10 mM HEPES, 0.1 mM CaCl₂, 4 mM Mg-ATP, 0.3 mM Na-GTP, 30 μ M (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohept-5,10-imine maleate (MK-801), and 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione were added to the external solution to block, respectively, *N*-methyl-D-aspartate and non-*N*-methyl-D-aspartate glutamate receptors. Conversely, to study spontaneous glutamate-mediated excitatory postsynaptic currents (sEPSCs), the recording pipettes were filled with internal solution of the following composition: 125 mM K⁺ gluconate, 10 mM NaCl, 1.0 mM CaCl₂, 2.0 mM MgCl₂, 0.5 mM BAPTA, 19 mM HEPES, 0.3 mM GTP, and 1.0 mM Mg-ATP, adjusted to pH 7.3 with KOH. Bicuculline (10 μ M) was added to the perfusing solution to block GABA_A-mediated transmission.

The detection threshold of sIPSCs, miniature GABA_A-mediated inhibitory postsynaptic currents, or sEPSCs was set at twice the baseline noise. The fact that no false events would be identified was confirmed by visual inspection of each experiment. Offline analysis was performed on spontaneous and miniature synaptic events recorded during fixed time epochs (5–10 samplings of 2- to 3-min duration each, recorded every 2–3 min), 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 were prepared 24 h after the last session of aggression.

Drugs. Corticosterone (from Sigma/RBI, Natick, MA) was administered subcutaneously 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; Alexis Biochemicals, San Diego, CA), alone or in combination with 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide (AM251) (6 mg/kg, dissolved in saline with DMSO 10% and Tween 80.5%; Tocris Bioscience, Bristol, UK) was injected intraperitoneally in nonstressed animals or immedi-

ately after the third of three sessions of stress. The injected volume was similar in single and combined treatments. In other experiments, URB597 (1 μ M dissolved in 1 μ l of DMSO) was also administered in vivo by an intracerebroventricular injection under stereotaxic coordinates (anterior, +0; lateral, +0.8; and dorsal, -2.4) and general anesthesia with 2,2,2-tribromoethanol (10 mg/ml, 1/27 of body weight) at the end of the three sessions of stress. Mice receiving subcutaneous, intraperitoneal, or intracerebroventricular injections of the appropriate volume of vehicle were used as controls.

Drugs used for the electrophysiological experiments were first dissolved in DMSO [AM251, (6*aR*)-*trans*-3-(1,1-dimethylheptyl)-6*a*,7,10,10*a*-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[*b,d*]pyran-9-methanol (HU210), and URB597] or water and then in the bathing artificial cerebrospinal fluid to the desired final concentration. The concentrations of the various drugs were chosen according to previous in vitro studies on corticostriatal brain slices (Maccarrone et al., 2008; Rossi et al., 2008) and were as follows: 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione, 10 μ M AM251, 1 μ M HU210, 30 μ M MK-801, 1 μ M tetrodotoxin [octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10*a*-dimethano-10*aH*-[1,3]dioxocino[6,5-*d*]pyrimidine-4,7,10,11,12-pentol] (all from Tocris Bioscience). Bicuculline (10 μ M) [(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) was from Sigma/RBI, and URB597 (1 μ M) was from Alexis Biochemicals. In the experiments with drugs dissolved in DMSO, the control samplings were obtained during DMSO and artificial cerebrospinal fluid 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 five to eight mice were used for a single electrophysiological experiment. Electrophysiological results from neurons recorded from the same animal were treated as a separate sample and averaged before calculating statistics. One to six neurons per animal were recorded. For data presented as the mean \pm 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 analyzed

by one-way analysis of variance followed by Tukey's honestly significant difference test. 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 Social Defeat Stress-Induced Anxiety. The antianxiety properties of intraperitoneal URB597 have been tested in experimental conditions not including social defeat stress (Kathuria et al., 2003; Patel and Hillard, 2006; Moreira et al., 2008; Scherma et al., 2008; Haller et al., 2009; Micale et al., 2009). Thus, mice were injected with a single intraperitoneal dose of URB597 or of vehicle at the end of the social defeat stress protocol, and the effects on both OFT and EPM were evaluated 24 h 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, because anxiety reduces the time spent in the center of the arena. Analysis of variance analysis showed a significant increase in anxiety in mice exposed to stress, because 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 group). 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 social defeat stress 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

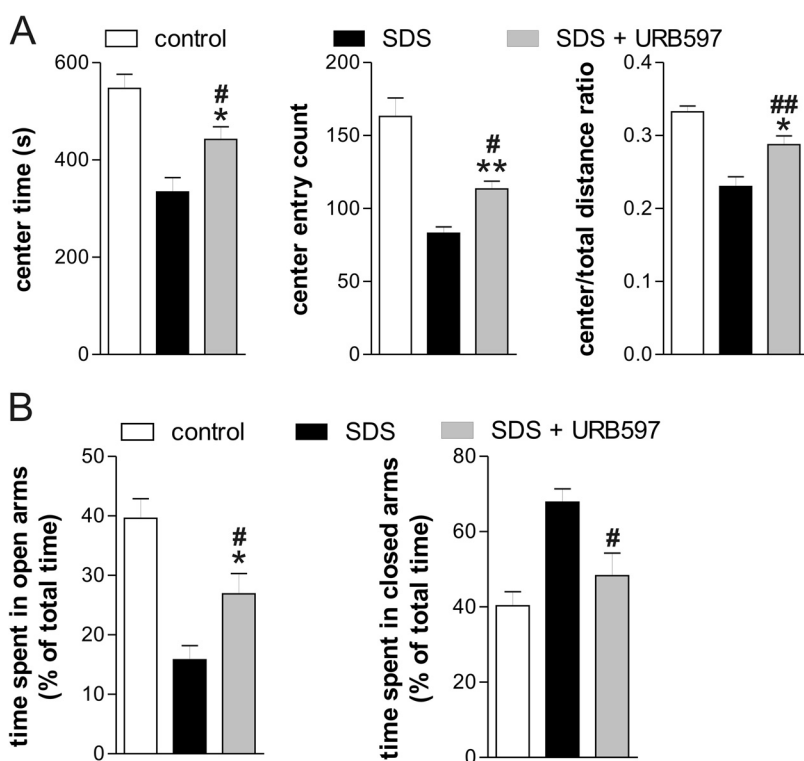


Fig. 1. FAAH inhibition protects from social defeat stress (SDS)-induced anxiety. A, the graphs show behavioral measures recorded at the OFT. Intraperitoneal administration of URB597 increased the activities in the center of arena (time spent in the center, count of the entries into the center) and the center/total distance ratio after social defeat stress. B, the graphs show behavioral measures recorded at the EPM. Intraperitoneal URB597 after social defeat stress increased the time spent in the open arms and reduced the time spent in the closed arms. *, $p < 0.05$, and **, $p < 0.01$ versus control; #, $p < 0.05$, and ##, $p < 0.01$ versus social defeat stress.

group). 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 Social Defeat Stress-Induced CB1R_(GABA) Blockade. Consistent with the behavioral data, we also found that a single intraperitoneal 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 ($n = 5$, $p < 0.01$), whereas it failed to affect GABA transmission after social defeat stress ($n = 7$, $p > 0.05$) (Rossi et al., 2008; De Chiara et al., 2010) (Fig. 2A). HU210, however, inhibited the frequency of sIPSCs in stressed mice treated with intraperitoneal URB597 ($n = 8$, $p < 0.01$), and it was ineffective in stressed mice only receiving the vehicle ($n = 6$, $p > 0.05$) (Fig. 2B). It is noteworthy that intraperitoneal URB597 ($n = 5$) failed to alter HU210-mediated sIPSC inhibition in nonstressed mice ($p < 0.05$ compared with pre-HU210 values, and $p > 0.05$ compared with HU210 effects in mice receiving intraperitoneal 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 social defeat stress in these mutants, as seen in response to intraperito-

neal URB597. In nonstressed 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 with pre-HU210 values). After social defeat stress, however, HU210 was still able to reduce striatal sIPSCs in FAAH(−/−) mice ($n = 5$, $p < 0.01$), whereas it was ineffective in control animals ($n = 5$, $p > 0.05$) (Fig. 2, D and 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 URB597. URB597, which significantly increases AEA but not 2-arachidonoylglycerol levels in striatal slices (Maccarrone et al., 2008), failed to affect sIPSC frequency ($n = 6$, $p > 0.05$) (Fig. 3A) and amplitude ($n = 6$, $98 \pm 3.1\%$ compared with predrug, $p > 0.05$; data not shown), whereas it significantly reduced sEPSC frequency ($n = 6$, $p < 0.01$, Fig. 3B). sEPSC amplitude was conversely unaltered by URB597 ($n = 6$, $p > 0.05$, $101 \pm 2.8\%$ compared with predrug, $p > 0.05$; data not shown). Inhibition of CB1Rs with the selective antagonist AM251 failed to alter per se sEPSC frequency ($n = 6$, $p > 0.05$, $97 \pm 3.3\%$ compared with predrug, $p > 0.05$; data not shown) but fully prevented the action of URB597 on sEPSC frequency ($n = 6$, $p > 0.05$), indicating that endogenous AEA preferentially

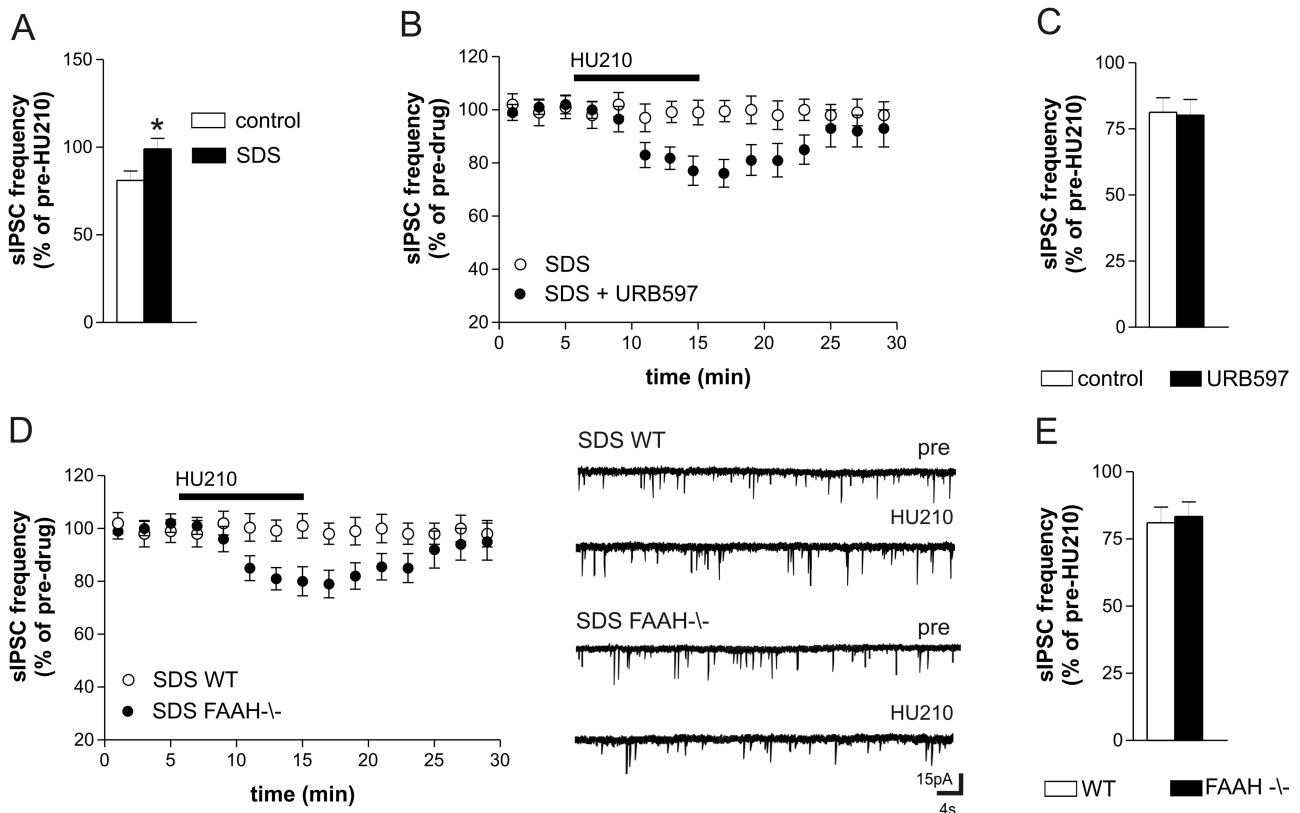


Fig. 2. FAAH inhibition protects from social defeat stress (SDS)-induced CB1R_(GABA) blockade. **A**, stimulation of CB1Rs with HU210 reduced sIPSC frequency in control mice. This effect was fully abolished in neurons from mice exposed to social defeat stress. **B**, intraperitoneal URB597 was able to rescue the effect of HU210 on sIPSC frequency in mice exposed to social defeat stress. **C**, intraperitoneal 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 social defeat stress, whereas 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 social defeat stress are shown at right. **E**, in FAAH(−/−) mice, HU210-induced inhibition of sIPSC frequency was similar to that observed in WT mice. *, $p < 0.05$ versus control.

activates presynaptic CB1Rs_(Glu) after 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 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 with predrug values for both experimental groups). We therefore analyzed the effects of AM251 on CB1Rs_(Glu) in control and FAAH(−/−) mice. AM251 failed to alter the frequency of sEPSCs in WT mice ($n = 6$ and $p > 0.05$ compared with predrug values), whereas it significantly increased sEPSC frequency in FAAH(−/−) mice ($n = 5$ and $p < 0.01$ compared with predrug 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 (data not shown).

FAAH Inhibition Protects from Stress Independent of Glucocorticoid Activity. FAAH inhibition reduces plasma corticosterone concentrations after stress (Patel et al., 2004), implying that the preserved activity of CB1Rs_(GABA) in FAAH(−/−) mice exposed to social defeat stress might be a consequence of reduced glucocorticoid activity in the brain. Therefore, systemic corticosterone administration results in CB1R_(GABA) blockade in nonstressed animals (Rossi et al., 2008). Thus, we administered corticosterone in FAAH(−/−) mice to determine whether this treatment was able to block striatal CB1Rs_(GABA) in these mice. Corticosterone treatment fully prevented HU210-induced inhibition of sIPSC frequency in control mice ($n = 6$, $p > 0.05$), whereas 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 CB1Rs_(GABA) after stress (Fig. 4A).

Selective Inhibition of FAAH in the Brain Protects CB1Rs_(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 CB1R_(GABA) activity in these mice does not rely on the inhibition of the hypothalamic-pituitary-adrenal axis by FAAH inhibition. Rather, these findings suggest that FAAH inhibition protects CB1Rs_(GABA) from the effects of both stress and corticosterone by acting centrally.

Thus, we measured HU210 effects in mice receiving URB597 through a single intracerebroventricular injection at the end of the social defeat stress protocol. As with intraperitoneal URB597, HU210 inhibited sIPSC frequency in social defeat stress mice treated with intracerebroventricular URB597 ($n = 6$, $p < 0.01$) but not with intracerebroventricular 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 antianxiety effects of URB597. Blockade of CB1R with AM251 prevented the anxiolytic properties of URB597. In fact, combined intraperitoneal URB597 plus AM251 administration after social defeat stress 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 group) 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 group) (Fig. 5, A and B). AM251 also blocked URB597 effects on striatal sIPSCs recorded from

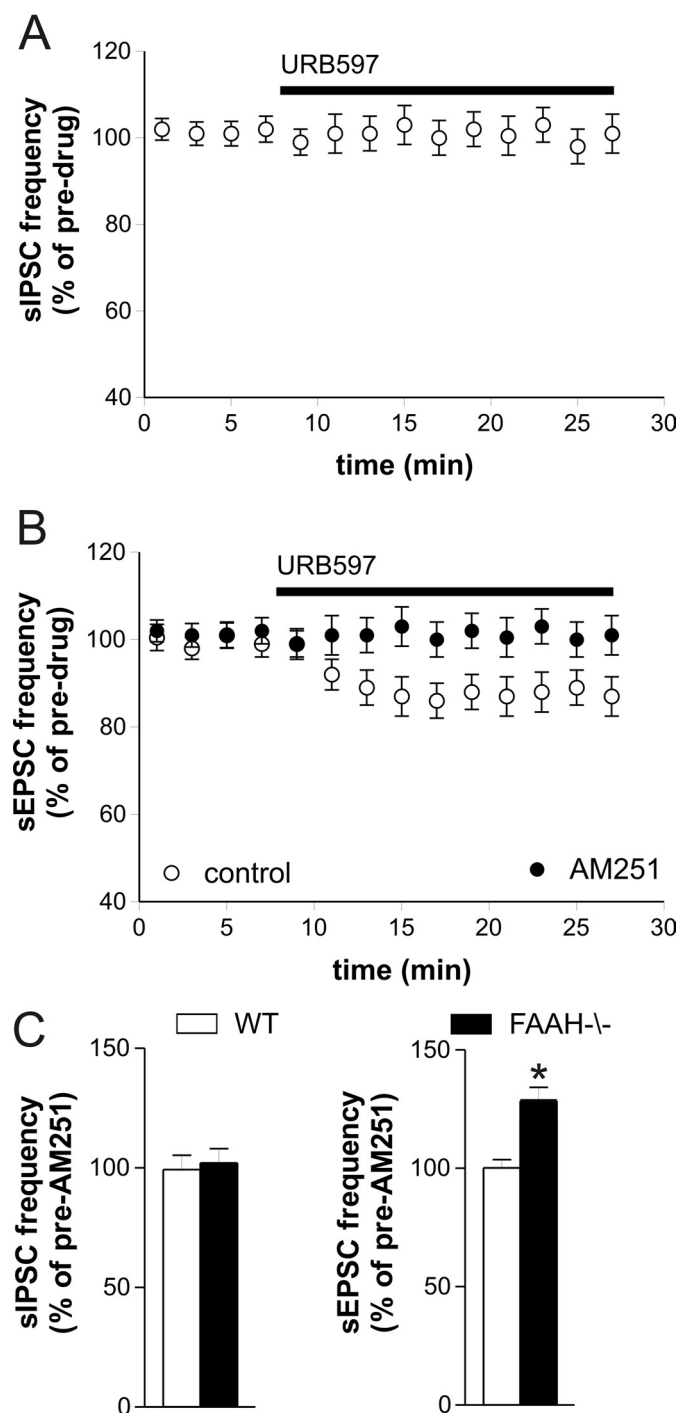


Fig. 3. FAAH inhibition results in CB1R_(Glu) but not CB1R_(GABA) activation. **A**, bath application of URB597 failed to affect sIPSC frequency. **B**, bath application of URB597 inhibited sEPSC frequency. Blockade of CB1Rs with AM251 blocked the effects of URB597 on sEPSCs. **C**, AM251 did not increase striatal sIPSC frequency in WT and in FAAH(−/−) mice. On the other hand, it significantly increased sEPSC frequency in FAAH(−/−) mice. *, $p < 0.05$ versus control.

stressed animals. In fact, URB597 plus AM251 failed to preserve the HU210-induced inhibition of sIPSC frequency ($n = 5$, $p > 0.05$ compared with 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 an 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 Δ^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), because 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 down-regulation of striatal CB1Rs_(GABA), such as after the social defeat stress 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 nonstressed 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 after FAAH inhibition) and CB1Rs_(GABA) (preserved from stress-induced down-regulation after FAAH inhibition) might play a role in mood control. This interaction might have physiological roles other than the control of social stress effects, because 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 probably regulates synaptic activity in multiple physiological and pathological conditions, including systemic inflammatory (Beishuizen and Thijs, 2003; Bornstein et al., 2008), 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

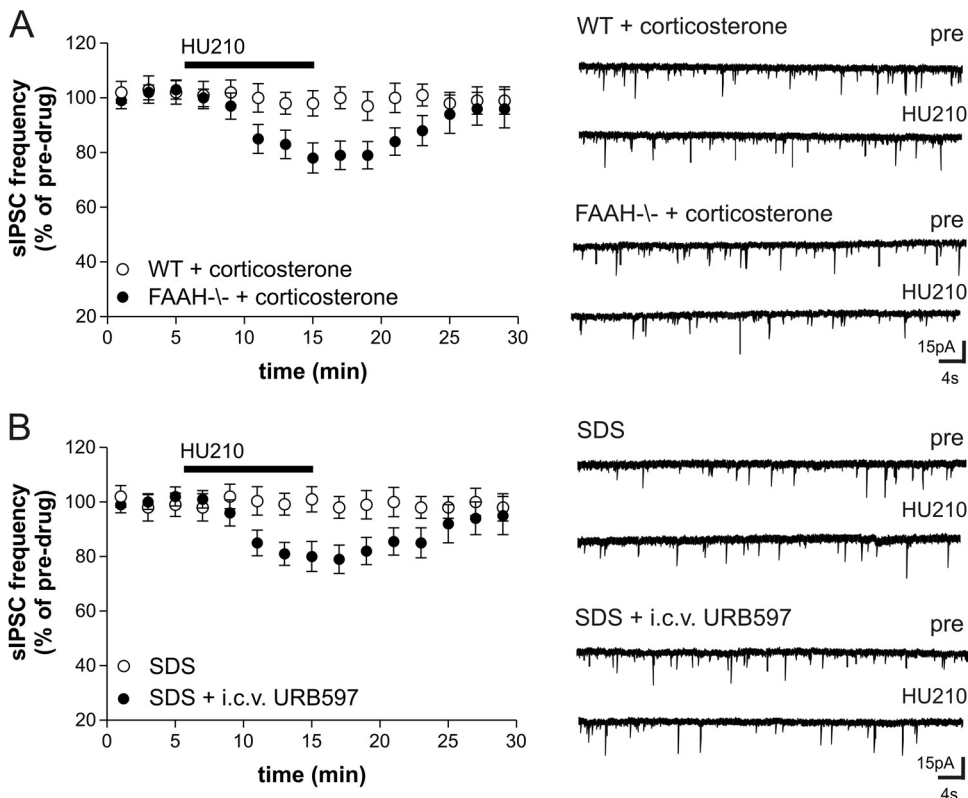


Fig. 4. FAAH inhibition protects from stress independently of glucocorticoid activity. **A**, corticosterone treatment mimicked the stress effects on HU210-induced reduction of sIPSC frequency in WT mice, whereas it was ineffective in FAAH^{-/-} mice. Examples of voltage-clamp recordings of sIPSC before and during the application of HU210 in WT and FAAH^{-/-} mice treated with corticosterone are shown on the right. **B**, the graph shows that intracerebroventricular URB597 was able to rescue the effect of HU210 on sIPSC frequency after social defeat stress (SDS).

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, 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. It is noteworthy that 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 long-term 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 and Isaacson, 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 antianxiety effects of systemic FAAH inhibition probably 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; Ganon-Elazar and Akirav, 2009; Hill et al., 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 emo-

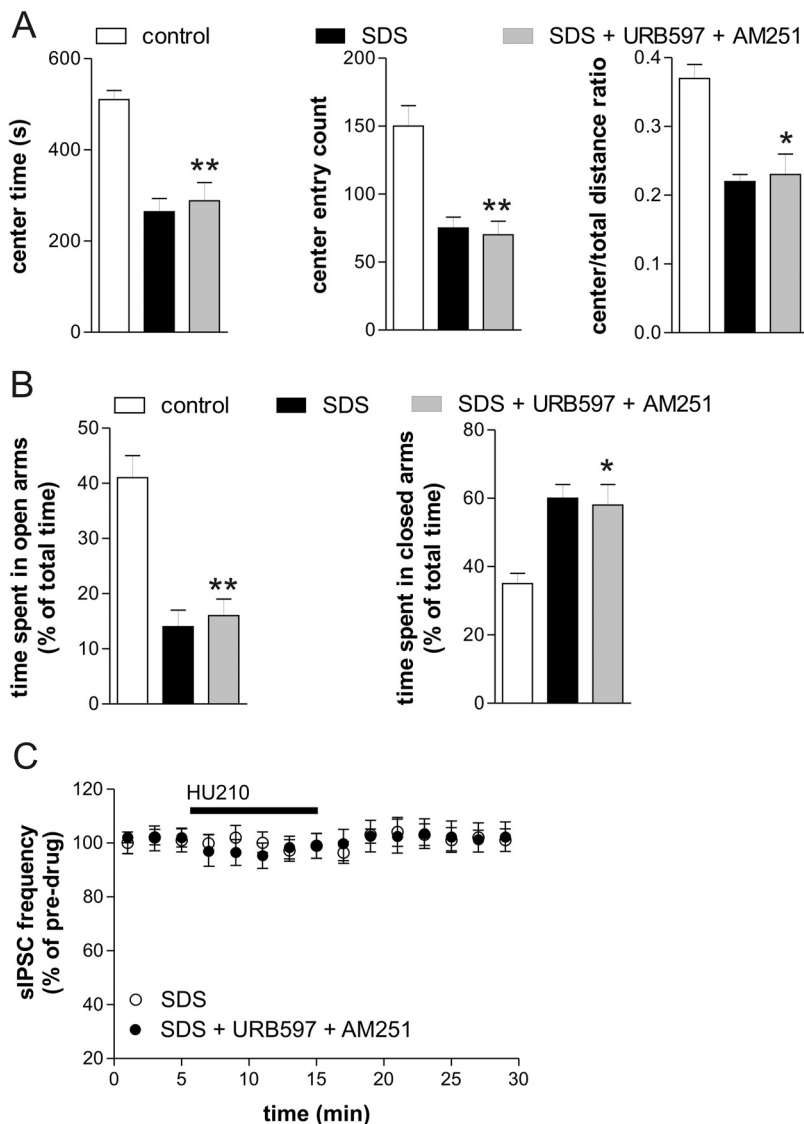


Fig. 5. CB1Rs are involved in the antianxiety effects of FAAH inhibition. **A**, the graphs show behavioral measures recorded at the OFT. Blockade of CB1Rs with AM251 fully prevented the anxiolytic properties of URB597 after social defeat stress (SDS). **B**, coadministration of URB597 and AM251 in stressed 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.

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

References

- Ade KK and Lovinger DM (2007) Anandamide regulates postnatal development of long-term synaptic plasticity in the rat dorsolateral striatum. *J Neurosci* **27**:2403–2409.
- Andersson M, Usiello A, Borgkvist A, Pozzi L, Dominguez C, Fienberg AA, Svenningsson P, Fredholm BB, Borrelli E, Greengard P, et al. (2005) Cannabinoid action depends on phosphorylation of dopamine- and cAMP-regulated phosphoprotein of 32 kDa at the protein kinase A site in striatal projection neurons. *J Neurosci* **25**:8432–8438.
- Avgustinovich DF, Kovalenko IL, and Kudryavtseva NN (2005) A model of anxious depression: persistence of behavioral pathology. *Neurosci Behav Physiol* **35**:917–924.
- Beishuizen A and Thijis LG (2003) Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *J Endocrinol Res* **9**:3–24.
- Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, et al. (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science* **311**:864–868.
- Bornstein SR, Engeland WC, Ehrhart-Bornstein M, and Herman JP (2008) Dissociation of ACTH and glucocorticoids. *Trends Endocrinol Metab* **19**:175–180.
- Bortolato M, Mangieri RA, Fu J, Kim JH, Arguello O, Duranti A, Tontini A, Mor M, Tarzia G, and Piomelli D (2007) Antidepressant-like activity of the fatty acid amide hydrolase inhibitor URB597 in a rat model of chronic mild stress. *Biol Psychiatry* **62**:1103–1110.
- Centonze D, Rossi S, Mercaldo V, Napoli I, Ciotti MT, De Chiara V, Musella A, Prosperetti C, Calabresi P, Bernardi G, et al. (2008) Abnormal striatal GABA transmission in the mouse model for the fragile X syndrome. *Biol Psychiatry* **63**:963–973.
- Cippitelli A, Cannella N, Braconi S, Duranti A, Tontini A, Bilbao A, Defonseca FR, Piomelli D, and Cicciocioppo R (2008) Increase of brain endocannabinoid anandamide levels by FAAH inhibition and alcohol abuse behaviours in the rat. *Psychopharmacology* **198**:449–460.
- Coddington E, Lewis C, Rose JD, and Moore FL (2007) Endocannabinoids mediate the effects of acute stress and corticosterone on sex behavior. *Endocrinology* **148**:493–500.
- Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, and Lichtman AH (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci USA* **98**:9371–9376.
- Cutolo M, Sulli A, Pizzorni C, Secchi ME, Soldano S, Serio B, Straub RH, Otsa K, and Maestroni GJ (2006) Circadian rhythms: glucocorticoids and arthritis. *Ann NY Acad Sci* **1069**:289–299.
- De Chiara V, Errico F, Musella A, Rossi S, Mataluni G, Sacchetti L, Siracusano A, Castelli M, Cavasinni F, Bernardi G, et al. (2010) Voluntary exercise and sucrose consumption enhance cannabinoid CB1 receptor sensitivity in the striatum. *Neuropsychopharmacology* **35**:374–387.
- Errico F, Santini E, Migliorini S, Borgkvist A, Centonze D, Nasti V, Carta M, De Chiara V, Prosperetti C, Spano D, et al. (2008) The GTP-binding protein Rhes modulates dopamine signalling in striatal medium spiny neurons. *Mol Cell Neurosci* **37**:335–345.
- Favilla C, Abel T, and Kelly MP (2008) Chronic Galphas signaling in the striatum increases anxiety-related behaviors independent of developmental effects. *J Neurosci* **28**:13952–13956.
- Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, and Piomelli D (2005) Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleylethanolamide deactivation. *J Pharmacol Exp Ther* **313**:352–358.
- Ganon-Elazar E and Akirav I (2009) Cannabinoid receptor activation in the basolateral amygdala blocks the effects of stress on the conditioning and extinction of inhibitory avoidance. *J Neurosci* **29**:11078–11088.
- Gerdeman G and Lovinger DM (2001) CB1 cannabinoid receptor inhibits synaptic release of glutamate in rat dorsolateral striatum. *J Neurophysiol* **85**:468–471.
- Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M, Cassano T, Morgese MG, Debonnel G, Duranti A, et al. (2005) Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proc Natl Acad Sci USA* **102**:18620–18625.
- Hack SP, Vaughan CW, and Christie MJ (2003) Modulation of GABA release during morphine withdrawal in midbrain neurons in vitro. *Neuropharmacology* **45**:575–584.
- Haller J, Barna I, Barsvari B, Gyimesi Pelczér K, Yasar S, Panlilio LV, and Goldberg S (2009) Interactions between environmental aversiveness and the anxiolytic effects of enhanced cannabinoid signaling by FAAH inhibition in rats. *Psychopharmacology (Berl)* **204**:607–616.
- Hariri AR, Gorka A, Hyde LW, Kimak M, Halder I, Ducci F, Ferrell RE, Goldman D, and Manuck SB (2009) Divergent effects of genetic variation in endocannabinoid signaling on human threat- and reward-related brain function. *Biol Psychiatry* **66**:9–16.
- Harvey VL and Stephens GJ (2004) Mechanism of GABA receptor-mediated inhibition of spontaneous GABA release onto cerebellar Purkinje cells. *Eur J Neurosci* **20**:684–700.
- Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, and Cullinan WE (2003) Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front Neuroendocrinol* **24**:151–180.
- Hill MN and Gorzalka BB (2006) Increased sensitivity to restraint stress and novelty-induced emotionality following long-term, high dose cannabinoid exposure. *Psychoneuroendocrinology* **31**:526–536.
- Hill MN, Karacabeyli ES, and Gorzalka BB (2007) Estrogen recruits the endocannabinoid system to modulate emotionality. *Psychoneuroendocrinology* **32**:350–357.
- Hill MN, McLaughlin RJ, Morrish AC, Viau V, Floresco SB, Hillard CJ, and Gorzalka BB (2009) Suppression of amygdalar endocannabinoid signaling by stress contributes to activation of the hypothalamic-pituitary-adrenal axis. *Neuropsychopharmacology* **34**:2733–2745.
- Hill MN, Patel S, Carrier EJ, Rademacher DJ, Ormerod BK, Hillard CJ, and Gorzalka BB (2005) Downregulation of endocannabinoid signaling in the hippocampus following chronic unpredictable stress. *Neuropsychopharmacology* **30**:508–515.
- Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley D, Mangieri R, Krey JF, Walker JM, Holmes PV, Crystal JD, et al. (2005) An endocannabinoid mechanism for stress-induced analgesia. *Nature* **435**:1108–1112.
- Howlett AC, Breivogel CS, Childers SR, Deadwyler SA, Hampson RE, and Porrino LJ (2004) Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacology* **47** (Suppl 1):345–358.
- Huang CC, Lo SW, and Hsu KS (2001) Presynaptic mechanisms underlying cannabinoid inhibition of excitatory synaptic transmission in rat striatal neurons. *J Physiol* **532**:731–748.
- Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, et al. (2003) Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* **9**:76–81.
- Kim KS, Lee KW, Baek IS, Lim CM, Krishnan V, Lee JK, Nestler EJ, and Han PL (2008) Adenylyl cyclase-5 activity in the nucleus accumbens regulates anxiety-related behavior. *J Neurochem* **107**:105–115.
- Lichtman AH and Martin BR (2005) Cannabinoid tolerance and dependence. *Handb Exp Pharmacol* **168**:691–717.
- Lister RG (1987) The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)* **92**:180–185.
- Maccarrone M, Rossi S, Bari M, De Chiara V, Fezza F, Musella A, Gasperi V, Prosperetti C, Bernardi G, Finazzi-Agrò A, et al. (2008) Anandamide inhibits metabolism and physiological actions of 2-arachidonoylglycerol in the striatum. *Nat Neurosci* **11**:152–159.
- Macfarlane DP, Forbes S, and Walker BR (2008) Glucocorticoids and fatty acid metabolism in humans: fuelling fat redistribution in the metabolic syndrome. *J Endocrinol* **197**:189–204.
- Mathew SJ and Ho S (2006) Etiology and neurobiology of social anxiety disorder. *J Clin Psychiatry* **67** (Suppl 12):9–13.
- McKinney MK and Cravatt BF (2005) Structure and function of fatty acid amide hydrolase. *Annu Rev Biochem* **74**:411–432.
- Micale V, Cristino L, Tamburella A, Petrosino S, Leggio GM, Drago F, and Di Marzo V (2009) Anxiolytic effects in mice of a dual blocker of fatty acid amide hydrolase and transient receptor potential vanilloid type-1 channels. *Neuropsychopharmacology* **34**:593–606.
- Moreira FA, Kaiser N, Monory K, and Lutz B (2008) Reduced anxiety-like behaviour induced by genetic and pharmacological inhibition of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH) is mediated by CB1 receptors. *Neuropharmacology* **54**:141–150.
- Murphy GJ and Isaacson JS (2003) Presynaptic cyclic nucleotide-gated ion channels modulate neurotransmission in the mammalian olfactory bulb. *Neuron* **37**:639–647.
- Musella A, De Chiara V, Rossi S, Prosperetti C, Bernardi G, Maccarrone M, and Centonze D (2009) TRPV1 channels facilitate glutamate transmission in the striatum. *Mol Cell Neurosci* **40**:89–97.
- Naidu PS, Varvel SA, Ahn K, Cravatt BF, Martin BR, and Lichtman AH (2007) Evaluation of fatty acid amide hydrolase inhibition in murine models of emotionality. *Psychopharmacology (Berl)* **192**:61–70.
- Patel S and Hillard CJ (2006) Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling. *J Pharmacol Exp Ther* **318**:304–311.
- Patel S, Roelke CT, Rademacher DJ, Cullinan WE, and Hillard CJ (2004) Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. *Endocrinology* **145**:5431–5438.
- Piomelli D (2003) The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci* **4**:873–884.
- Rademacher DJ and Hillard CJ (2007) Interactions between endocannabinoids and stress-induced decreased sensitivity to natural reward. *Prog Neuropsychopharmacol Biol Psychiatry* **31**:633–641.
- Reiman EM, Raichle ME, Robins E, Mintun MA, Fusselman MJ, Fox PT, Price JL, and Hackman KA (1989) Neuroanatomical correlates of a lactate-induced anxiety attack. *Arch Gen Psychiatry* **46**:493–500.
- Rogan MT, Leon KS, Perez DL, and Kandel ER (2005) Distinct neural signatures for safety and danger in the amygdala and striatum of the mouse. *Neuron* **46**:309–320.
- Rossi S, De Chiara V, Musella A, Kusayanagi H, Mataluni G, Bernardi G, Usiello A, and Centonze D (2008) Chronic psychoemotional stress impairs cannabinoid-receptor-mediated control of GABA transmission in the striatum. *J Neurosci* **28**:7284–7292.
- Rubino T, Realini N, Castiglioni C, Guidali C, Viganó D, Marras E, Petrosino S, Perletti G, Maccarrone M, Di Marzo V, et al. (2008) Role in anxiety behavior of the endocannabinoid system in the prefrontal cortex. *Cereb Cortex* **18**:1292–1301.
- Scherma M, Medalie J, Fratta W, Vavilov SK, Makriyannis A, Piomelli D, Mikics E, Haller J, Yasar S, Tanda G, et al. (2008) The endogenous cannabinoid anandamide has effects on motivation and anxiety that are revealed by fatty acid amide hydrolase (FAAH) inhibition. *Neuropharmacology* **54**:129–140.
- Seckl JR and Meaney MJ (2004) Glucocorticoid programming. *Ann NY Acad Sci* **1032**:63–84.
- Szabo B, Dörner L, Pfreundtner C, Nörenberg W, and Starke K (1998) Inhibition of

- GABAergic inhibitory postsynaptic currents by cannabinoids in rat corpus striatum. *Neuroscience* **85**:395–403.
- Yoo HK, Kim MJ, Kim SJ, Sung YH, Sim ME, Lee YS, Song SY, Kee BS, and Lyoo IK (2005) Putaminal gray matter volume decrease in panic disorder: an optimized voxel-based morphometry study. *Eur J Neurosci* **22**:2089–2094.
- Zhang HT, Huang Y, Masood A, Stolinski LR, Li Y, Zhang L, Dlaboga D, Jin SL, Conti M, and O'Donnell JM (2008) Anxiogenic-like behavioral phenotype of mice

deficient in phosphodiesterase 4B (PDE4B). *Neuropsychopharmacology* **33**:1611–1623.

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