SUMMARY

The endocannabinoid system plays a fundamental role in the regulation of synaptic transmission. Exposure to stressful or rewarding events triggers synaptic adaptations in many brain areas. The activity of the endocannabinoid system in stress-responsive neural circuits and central reward pathway suggests that it may be involved in the behavioural responses and synaptic effects typical of stress, drug addiction an other forms of reward-based behaviors. In the present study, by means of electrophysiological recordings we found that social defeat stress, induced in mice by exposure to aggression, and natural rewards such as running wheel and sucrose consumption, alter in opposite way the cannabinoid CB1 receptor-mediated control of synaptic transmission in the striatum. The striatum plays a central role in motor, cognitive and emotional functions modulated by stress and rewarding agents, and contains high levels of cannabinoid receptors controlling both excitatory and inhibitory synaptic transmission .

We found that the presynaptic inhibition of GABAergic inhibitory postsynaptic currents induced by the cannabinoid CB1 receptor agonist HU210 was abolished after chronic stress exposure, whereas it was remarkably potentiated after running wheel and sucrose consumption. In contrast, the sensitivity of glutamate synapses to CB1 receptor stimulation was unaltered, as well as that of GABA synapses to the stimulation of other presynaptic receptors such as GABAB.

The alterations of cannabinoid CB1 receptor-mediated responses were slowly reversible and were also detectable following the mobilization of endocannabinoids by metabotropic glutamate receptor 5 stimulation. Finally, we found that the up-regulation of cannabinoid transmission induced by wheel running or sucrose played a crucial role in the protective effects of these environmental manipulations against the motor and synaptic consequences of stress.

Since BDNF also play a role in the emotional consequences of stress and of rewarding experiences, we have extended our study to address the functional interplay between BDNF and cannabinoid CB1 receptors in the striatum. We found that BDNF potently inhibits CB1 receptor function in the striatum. The action of BDNF on CB1 receptor function was tyrosine kinase dependent, and was

complete even after receptor sensitization with behavioral manipulations activating the reward system.

BDNF-mediated regulation of striatal CB1 receptors might have relevant roles in cognitive and behavioral functions and targeting cannabinoid CB1 receptors or endocannabinoid metabolism might be a valuable option to treat stress-associated neuropsychiatric conditions.

INTRODUCTION

The endocannabinoid system

Cannabis sativa is a psychoactive substance used recreationally worldwide for its relaxing and stress alleviating properties. These emotional alterations are mostly mediated by the ability of its constituent Δ9-tetrahydrocannabinol (Isbell *et al.*, 1967) to interact with specific cannabinoid receptors throughout the brain. Two receptors have been characterized and cloned to date, CB1 and CB2 receptors. They are both G-protein-coupled receptors, that activate $Galpha_{i/o}$ proteins resulting in inhibition of adenylyl cyclase activity (Felder *et al.*, 1998; Piomelli *et al.*, 2003). In addition, they activate mitogen-activated-protein kinases and ion channels as A-type and inwardly rectifying potassium channels, and inhibit calcium channels activated by membrane depolarizations (Felder *et al.*, 1998; Piomelli *et al.*, 2003; Van der Stelt *et al.*, 2003).

The CB1 receptor is the predominant cannabinoid receptor in the central nervous system (Herkenham *et al.*, 1991; Moldrich *et al.*, 2000), but, at lower expression levels, it has been found also in the periphery such as blood vessels, immune cells and reproductive tissues (Parolaro *et al.*, 1999; Hillard *et al.*, 200; Habayeb *et al.*, 2008). The CB2 receptor is located mainly in immune cells, such as macrophages (Parolaro *et al.*, 1999) and microglia (Cabral *et al.*, 2005), but evidence exists that it is also expressed in neurons of the brainstem, cortex and cerebellum (Van Sickle *et al.*, 2005; *Gong et al.*, 2008). In addition to these targets, some cannabinoids may exhibit affinity for other receptor subtypes, such as transient receptor potential vanilloid 1 (TRPV1) receptors (Ross 2003) or transient receptor potential ankjrin 1 receptors (De Petrocellis *et al.*, 2008), peroxisome-proliferator-activated receptors (Sun *et al.*, 2006) and non-CB1/CB2 G-protein-coupled receptors GPR55 (Ryberg *et al.*, 2007).

Several endogenous ligands of the cannabinoid receptors, termed endocannabinoids, have been isolated from brain tissues, anandamide (AEA, Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG, Sugiura *et al.*, 1997) being the best characterized. Both endocannabinoids are synthesized

preferentially post-synaptically by cleavage of phospholipidic groups by specific enzymes, such as dyiacylglycerol lipase (DAGL) for 2-AG, and a Ca^{2+}-dependent N-acyltransferase together with N-acylphosphatidylethanolamine specific phospholipase D (NAPE-PLD) for AEA (Di Marzo *et al.*, 1998). Endocannabinoid levels, therefore, are maintained by catabolic enzymes, and namely by the fatty acid amide hydrolase (FAAH) for AEA and by the monoacylglyceride lipase (MAGL) for 2-AG (Ueda 2002, Dinh *et al.*, 2002) even if a recent study suggests the involvement of FAAH in controlling 2-AG levels (Di Marzo and Maccarrone, 2008).

The endocannabinoids, synthesized "on demand" in response to increased neuronal excitation or increased intracellular calcium, act in a retrograde manner to activate the presynaptic CB1 receptors and to inhibit neurotrasmitter release (Bisogno *et al.*, 2005; Basavarajappa *et al.*, 2007). Endocannabinoid-mediated retrograde control of synaptic activity has also been recently demonstrated after activation of group I metabotropic glutamate receptors (Jung *et al.*, 2005; Centonze *et al.*, 2007) or dopamine D2 receptors (Centonze *et al.*, 2004; Melis *et al.*, 2004). The neurophysiological consequences of the activation of CB1 receptors depend on the localization of these receptors in various brain regions and the excitatory or inhibitory pathways being stimulated. Hence, the clinical potential of cannabinoid drugs is vast.

The striatal endocannabinoid system

The basal ganglia form a subcortical station where information coming from the cortex is collected, integrated ant then sent back to modulate motor programs and cognitive processes. The striatum is the main receiving area of the basal ganglia, mostly consisting of GABAergic medium spiny neurons, which account for the 95% of its population. These cells receive glutamatergic excitatory input from the cortex, GABAergic inhibitory input from axon collaterals and striatal interneurons and modulatory input from midbrain dopaminergic neurons.

Accordingly, striatal GABAergic principal neurons, besides inhibiting basal ganglia output nuclei, form functional synapses through their recurrent axon collaterals, establishing a feedback control

over striatal neuron activity (Tunstall *et al*, 2002; Guzman *et al*, 2003; Koos *et al*, 2004; Gustafson *et al*, 2006). Inputs from GABAergic interneurons are another important source of synaptic inhibition of projection neurons, giving rise to a feedforward inhibitory pathway that is independent of striatal output (Plenz 2003; Tepper *et al*, 2004; Gustafson *et al*, 2006). Both the feedback and the feedforward intrastriatal GABAergic pathways are likely modulated by cannabinoid CB1 receptors. In line with this conclusion, CB1 receptors are expressed at very high concentrations on both axon collaterals of striatal projection neurons and on GABA interneurons (Herkenham *et al*, 1991; Hohmann and Herkenham, 2000).

CB1 receptor are also expressed on corticostriatal glutamatergic fibers (Huang *et al.*, 2001).

In the striatum, stimulation of cannabinoid CB1 receptors presynaptically reduces both glutamatergic and GABAergic transmission (Gerdeman and Lovinger, 2001; Huang *et al.*, 2001; Centonze *et al.*, 2007b; Maccarone *et al.*, 2008). Interaction between the inhibitor and excitatory inputs controls the output of the striatum to the substantia nigra and globus pallidus (Calabresi *et al*, 1991).

Stress and endocannabinoid system

In vitro data demonstrated that both AEA and 2-AG contents increase in hypothalamic tissue after application of glucocorticoids (Di *et al.*, 2005), suggesting that stress could result in a rapid induction of endocannabinoid signalling. Conversely, acute stress exposure (30 min of restraint stress) failed to alter endocannabinoid levels in the ventral striatum (Rademacher *et al.*, 2008), and decreased them in other neural structures (Rademacher *et al.*, 2008; Patel *et al.*, 2004).

Other studies have addressed the regulation of striatal endocannabinoid levels under conditions of chronic stress. The effects of repeated homotipic stress have been examined in male mice exposed to 5-10 daily sessions of restraint stress for 30 min (Rademacher *et al.*, 2008). In this study, an opposite pattern of AEA alteration has been described, since AEA content was significantly elevated in the ventral striatum following 10 but not 7 days of repeated restraint stress, whereas 2-

AG content was reduced following 7 but not 10 days of this treatment. Since it has been hypothesized that glutamatergic synapses are primarily controlled by AEA while GABAergic terminals by 2-AG (Freund *et al.,* 2003; Musella *et al.,* 2009), and it has demonstrated that AEA can inhibit 2-AG-mediated control of GABAergic transmission (Maccarone *et al.,* 2008), the changes of the two endocannabinoids in opposite direction could result in enhanced GABAergic tone of synaptic transmission. The implications of this change are not clear at this stage, but it could reflect the habituation process of behavioural responses than usually occurs after repeated homotipic stress.

A different pattern of endocannabinoid system-stress interaction has been seen when animals are exposed to chronic unpredictable and varying stress regimens. These protocols are associated with hypersecretion of glucocorticoids and lack of habituation (Herman *et al.,* 1995). In the study by Hill and coworkers (Hill *et al.,* 2007), exposure of rats for 21 days to chronic unpredictable stress (CUS) reduced tissue content of AEA in ventral striatum and in other brain regions. This finding stands in contrast to an earlier study (Bortolato *et al.,* 2007), in which CUS failed to affect AEA and 2-AG contents in the ventral striatum. It is likely that methodological differences are responsible for this discrepancy.

Reduction of cannabinoid activity could contribute to the development of depression, by promoting maladaptive responses to prolonged stress. In fact, the CUS model is considered as a valid model of depression, eliciting abnormal behavioural and physiological responses reminiscent of those observed in depressed patients: alterations in feeding and body weight, enhanced fearfulness, impaired sleep architecture, and inadequate self-care (Willner P. 2005).

The expression of cannabinoid CB1 receptors during conditions of stress has been poorly investigated so far. Rademacher and coworkers (Rademacher *et al*., 2008) reported that exposure to 10 days of repeated stress failed to affect CB1 receptor binding in the ventral striatum. In contrast, exposure to CUS seems to lead to different effects, since CB1 receptor binding site density was reduced by CUS in the ventral striatum (Hill *et al*., 2007; Hillard *et al*., 2006). Furthermore, a recent report showed that no change occurred in striatal CB1 mRNA after 70 days of chronic mild

unpredictable stress (Bortolato *et al*., 2007). Because of the diverse methodological approaches, it is difficult to compare the alterations of the endocannabinoid system that follow chronic homotypic stress and those induced by chronic heterotypic stress. However, the existing data seem to suggest that disruption of the endocannabinoid signalling may prevent adaptive responses and compromise the habituation process during CUS. In line with this, the antagonism of the CB1 receptor has been found to partially reverse the habituation of behavioural activation and neuroendocrine responses in repeatedly stressed mice (Patel *et al*., 2005). Moreover, mice lacking cannabinoid CB1 receptors showed a complete absence of habituation of freezing behaviours when exposed repeatedly to an audiogenic stressor (Kamprath *et al*., 2006). Thus, deficiencies in the endocannabinoid signalling could prevent the normally occurring adaptation to a repeatedly presented aversive stimulus.

Natural reward and endocannabinoid system

Studies with drugs of abuse suggested that cannabinoid receptors are involved in the regulation of the central reward system. Accordingly, inactivation of cannabinoid CB1 receptors attenuates the rewarding effects of cannabinoids (Ledent *et al*, 1999), opiates (Ledent *et al*, 1999; Martin *et al*, 2000**;** Cossu *et al*, 2001), and cocaine (Chaperon *et al*, 1998), while stimulation of these receptors elicits relapse not only to cannabinoid consumption but also to cocaine, heroin, alcohol and methamphetamine (Fattore *et al*, 2007; Higuera-Matas *et al*, 2008).

We have recently reported that cocaine-induced conditioned place preference (CPP) is associated with hypersensitivity of striatal GABA synapses to the stimulation of cannabinoid CB1 receptors (Centonze *et al*, 2007a). This finding raises the possibility that other forms of reward-based behaviors may rely on the sensitization of cannabinoid CB1 receptor-mediated transmission in this brain area. Running wheel activity has strong rewarding and reinforcing properties in rodents, and shares many neurochemical and behavioral characteristics with drug-induced reward situations, through the modulation of striatal neuron activity (Werme *et al*, 2000; 2002; Lett *et al*, 2001; de Visser *et al*, 2007). It is therefore conceivable that this environmental manipulation mimics the

effects of cocaine on cannabinoid-mediated control of striatal synaptic transmission and, in support of this hypothesis, it has been reported that voluntary running wheel exercise activates striatal dopamine (DA) signaling (El Rawas *et al*, 2009), and increases some behavioral and metabolic effects of cannabinoid CB1 receptor blockade (Zhou and Shearman, 2004).

Sweet foods and drinks also have intense rewarding properties (Lenoir *et al*, 2007), and many commonalities exist between overconsumption of sugars and drug addiction (Levine *et al*, 2003; Kelley, 2004; Volkow and Wise, 2005). Accordingly, both sweet tastants (Mark *et al*, 1991; Hajnal *et al*, 2004) and drugs of abuse (Di Chiara and Imperato, 1988; Pontieri *et al*, 1996) stimulate DA signaling in the striatum, and both cross-tolerance (Lieblich *et al*, 1983; d'Anci *et al*, 1996) and cross-dependence (Rudski *et al*, 1997; Kanarek *et al*, 1997; Colantuoini *et al*, 2004) have been observed between sugars and drugs of abuse. Furthermore, the endocannabinoid system plays a key role in the rewarding properties of palatable foods (Cota *et al*, 2003; Mahler *et al*, 2007), and cannabinoid CB1 receptor blockade decreases motivation for sweet foods, while activation of these receptors increases it (Simiand *et al*, 1998; Cota *et al*, 2003; Ward and Dykstra, 2005; Mahler *et al*, 2007).

BDNF and endocannabinoid system

Brain-derived neurotrophic factor (BDNF) is a widely expressed, activity-regulated secretory protein with pleiotropic actions within the central nervous system. Besides its role in promoting neuronal proliferation, differentiation, migration, and survival, BDNF is a key regulator of synaptic transmission and plasticity in the adult brain (Carvaho *et al.*, 2008; Waterhouse and Xu, 2009). These composite actions are likely to mediate the neuroprotective effects of BDNF (Castrén, 2004; Lu *et al*., 2005; Hennigan *et al.*, 2007), as well as its complex effects on cognition and mood (Lu *et al.*, 2005; Martinowich *et al*., 2007). Signaling through cannabinoid CB1 receptors is also emerging as a critical determinant in neuroprotection (Martínez-Orgado *et al*., 2007; Galve-Roperh *et al*., 2008), learning and memory (Horder *et al*., 2009; Puighermanal *et al*., 2009), and emotional control

(Aso *et al*., 2008; Juhasz *et al*., 2009; Moreira *et al*., 2009), raising the possibility that BDNF and CB1Rs interact to regulate multiple functions in the brain.

At present, only indirect evidence suggests a BDNF-CB1 receptors interaction. Accordingly, BDNF levels are decreased in the brain of mice lacking CB1 receptors (Aso *et al*., 2008), while activation of these receptors increases BDNF in rodents (Butovsky *et al*., 2005), and in humans (D'Souza *et al*., 2009). Furthermore, BDNF release triggered by CB1 receptors stimulation mediates the neuroprotective effects of cannabinoids (Khaspekov *et al*., 2004). While these data indicate that stimulation of CB1 receptors promotes BDNF release and activity, the effects of BDNF activity on CB1 receptor function are fully unexplored.

In the striatum, BDNF and CB1 receptors seem to act in opposite ways to regulate emotionality. Intrastriatal infusion of BDNF, in fact, elicits a depressive behavior (Eisch *et al*., 2003), as also does a stress protocol which causes the down-regulation of striatal CB1 receptors (Berton *et al*., 2006). Furthermore, the anxious-depressive behavior induced by social defeat stress is abolished by either blockade of BDNF signaling in this brain area (Berton *et al*., 2006). These data argue against a synergistic action of CB1Rs and BDNF, and rather suggest that BDNF contrasts the activity of CB1Rs.

MATERIALS AND METHODS

For the experiments were used male C57/bl6 mice (6-7 weeks old) and mice lacking one copy of the BDNF gene (BDNF+/-) and expressing reduced activity of BDNF (Jeanblanc *et al*., 2006; Saylor and McGinty, 2008). All animals were housed, four per cage, on a 12 h light/dark cycle with lights on at 06:00 h and controlled (22-23°C) temperature.

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).

Natural reward pardigm

C57/bl6 mice were randomly allocated in the control, running wheel or sucrose groups. For each experimental group, a numerically comparable group of control animals was constituted by age-matched mice never exposed to running wheel and drinking normal water.

To study the effects of voluntary exercise, mice were housed in a cage equipped with a running wheel for 1, 3, 7, 15 or 30 days. Another group of mice was reared in control cages and were allowed to consume *ad libitum* a drinking fluid containing sucrose (3% in tap water) for 1, 3, 7 or 15 days. In some mice exposed for 7 days to sucrose, sucrose-containing solution was replaced with normal water for 1, 3, or 7 days before the electrophysiological experiments. Furthermore, in some mice exposed for 15 days to running wheel, the enriched cage was replaced with a normal one for 1, 3 or 7 days before the electrophysiological experiments.

Social defeat

Chronic psychoemotional stress induced by negative experience of social defeats in intermale confrontations is well known to lead to the development of anxious-depressive symptoms in male mice. In the present work, psycoemotional stress was induced by using a protocol already published

by Avgustinovich DF *et al*. (2005) and by Berton *et al*. (2006). Briefly, C57B6 7/8 weeks old mice were subjected to daily bouts for 10 min 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 1, 3 or 7 consecutive days.

Elevated plus maze

The elevated plus maze 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 maze was wiped clean.

Sucrose preference

The test was performed as previously described (Sonnier *et al*, 2007). Male C57/bl6 mice (n = 11 per sucrose concentration), placed in individual cages 2 weeks before the test, were submitted to a water versus sucrose two-bottle preference test. Increasing sucrose solutions were used (0.75% and 3%). Each concentration was presented in consecutive 2-day blocks. The solutions were available 23 h/day. During the remaining 1 h, the volumes consumed were measured and the bottles refilled. The left–right positions of the sucrose and water were alternated for each concentration (to control for the preference of some mice for a particular side). Total intakes (in ml) were averaged . Preference for sucrose (%) was calculated as the ml of sucrose solution drank over the total drink intake (e.g. [sucrose/sucrose + water] x 100). Significance was calculated by one-way ANOVA (sucrose concentration effect), followed by Fisher's *post-hoc* analysis.

Open field test

Twenty-four hours after the last event of sensory aggression, mice were subjected to the open-field test to compare their motor responses to those of control (not stressed and standard-housed) animals (n=13). Motor response was also addressed in the open-field in rewarded (15 days running wheel (n=9) and 7 days sucrose (n=9)), not stressed mice.

The open-field paradigm assesses motor activity of animals in an averse, stressful environment. This protocol was performed as previously reported (Errico *et al*, 2008). 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 center/total distance ratio used as an index of anxiety-related behavior.

Electrophysiology

Corticostriatal coronal slices (200 µm) were prepared from tissue blocks of the mouse brain with the use of a vibratome (Centonze *et al*., 2005; 2007a,b). A single slice was then transferred to a recording chamber and submerged in a continuously flowing artificial cerebrospinal fluid (ACSF) (32°C, 2-3 ml/min) gassed with 95% O_2- 5% CO_2. The composition of the control solution was (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 11 Glucose, 25 $NaHCO_3$. The striatum could be readily identified under low power magnification, whereas individual neurons were visualized in situ using a differential interference contrast (Nomarski) optical system. This employed an Olympus BX50WI (Japan) non-inverted microscope with x40 water immersion objective combined with an infra-red filter, a monochrome CCD camera (COHU 4912), and a PC compatible system for analysis of images and contrast enhancement (WinVision 2000, Delta Sistemi, Italy). Whole-cell patch clamp recordings were made with borosilicate glass pipettes (1.8 mm o.d.; 2-4 MΩ), in voltage-clamp mode, at the holding potential (HP) of –80 mV. Recording

pipettes were advanced towards individual striatal cells in the slice under positive pressure and, on contact, tight GΩ seals were made by applying negative pressure. The membrane patch was then ruptured by suction and membrane current and potential monitored using an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Whole-cell access resistances measured in voltage clamp were in the range of 5-20 MΩ.

In a set of experiment sigle slices were incubated for 1 hour in the presence of BDNF (10 ng/ml; first dissolved in water 10 µg/ml), lavendustin A (10 µM; first dissolved in DMSO 10 mM), or BDNF plus lavendustin A and were then transferred to the recording chamber.

To detect evoked (eIPSCs), 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. Only cells that exhibited stable frequencies in control (less than 20% changes during the control samplings) were taken into account. The rare

events with complex peaks were eliminated, because amplitudes of the single synaptic currents composing these events were difficult to determine.

Mice were killed for the electrophysiological evaluations immediately after exposure to running wheel or to sucrose. In other experiments, 24 hours was the time interval between the last session of stress and the electrophysiological recordings.

Statistical analysis

Throughout the text, "n" refers to the number of cells, unless otherwise specified. One to 6 neurons per animal were recorded. Each electrophysiological measure in each group of mice was obtained by pooling data from at least 6 different animals. 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 analyzed by one-way or two-way ANOVA followed by appropriate post-hoc comparisons (Tukey HSD or Student's *t*-test). The significance level was established at $p<0.05$. To determine differences between two cumulative distributions, the Kolmogorov-Smirnov test was used.

Drugs

In some experiments, RU486 (from Sigma-RBI, St. Louis, USA, 25 mg/Kg) was dissolved in 100 µl DMSO and injected intraperitoneally 5-10 min prior to each session of 3 consecutive days of stress. In other experiments, RU486 was emulsioned through sonication in 200 µl saline (0.9% NaCl). The data were not different between the two groups of experiments and were pooled together. Corticosterone (from Sigma-RBI, St. Louis, USA) was administrated 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). Mice receiving injections of the appropriate vehicle were used as controls.

A single dose of BDNF (1 µl) was also administered *in vivo* by a intracerebroventicular (i.c.v.) injection (0.2 µg/µl saline) 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).

Drugs used in slices for the electrophysiological experiments were first dissolved in DMSO (AM251, HU210) or water, then in the bathing ACSF to the desired final concentration. DMSO was used when appropriate in control experiments. The concentrations of the various drugs were chosen according to previous in vitro studies on corticostriatal brain slices (Centonze *et al*, 2007a,b), and were as follows: AM251 (10 µM), baclofen (3 µM, 10 µM), CNQX (10 µM), 3,5-DHPG (DHPG, 50 µM), HU210 (0.1 µM, 0.3 µM, 1 µM, 3 µM), MK-801 (30 µM), tetrodotoxin (TTX, 1 µM) (from Tocris, Bristol, UK). Bicuculline (10 µM) (from Sigma-RBI, St. Louis, USA). Unless otherwise specified, HU210 was applied at the concentration of 1 µM.

RESULTS

Behavioral effect of the social defeat and of the rewarding paradigms

The rewarding properties of running wheel (15 and 30 days) have already been established in previous studies. Rodents, for example, display CPP to an environment associated with wheel running (Iversen, 1993; Belke, 1997; Lett *et al*, 2000; de Visser *et al*, 2007), and exhibit withdrawal signs when access to the running wheels is denied (Hoffmann *et al*, 1987).

To evaluate the behavioral effects associated with social defeat (3 days) and with the exposure to sucrose-containing solution (7 and 15 days) we used the elevated plus maze paradigm and the a two-bottle preference test respectively.

Plus maze paradigm was used to verify the effectiveness of our social defeat protocol to induce an anxious state 24 h after the last aggression. All elevated plus maze measures showed significant difference between stress group (exposed animals; n=8) and control group (unexposed animals; n=8). When compared to unexposed mice, exposed animals showed a significant reduction in the time spent in the open arms (6.6±2.2% vs 17.5±4.5%; $p<0.05$) and an increase in the time spent in the closed arms (80.8±3.0% vs 67.2±4.5%; $p<0.05$) (not shown).

In order to see whether at the dose here employed sucrose elicits hedonic effects, we performed a two-bottle preference test. The behavioral results demonstrated that, at both concentrations tested (0.75% and 3%), sucrose induced in mice a robust rewarding response. Accordingly, one-way ANOVA indicated an overall impact of sucrose on drinking preference of mice [$F_{(2, 20)} = 46.391$, $p < 0.0001$]. Moreover Fisher's *post-hoc* comparisons showed a similar pleasurable effect of both doses on mice (water *vs* 0.75%: $p < 0.0001$; water *vs* 3%: $p < 0.0001$; 0.75% *vs* 3% sucrose: $p > 0.1$) (not shown).

Effects of HU210 on GABA transmission in stressed mice and in mice exposed to running wheel or sucrose

As described (Centonze *et al*., 2007a,b), application of the cannabinoid CB1 receptor agonist HU210 (10 min, n=14) significantly ($p<0.01$) reduced the frequency of sIPSCs in control striatal neurons, an effect prevented by pre-incubating the slices with the selective antagonist of CB1 receptors AM251 (n=6 and $p>0.05$) (Fig. 1A). In neurons from mice receiving 3 (n=28) and 7 days (n=13) of psychoemotional stress, HU210 effects were completely abolished ($p>0.05$) (Fig. 1B).

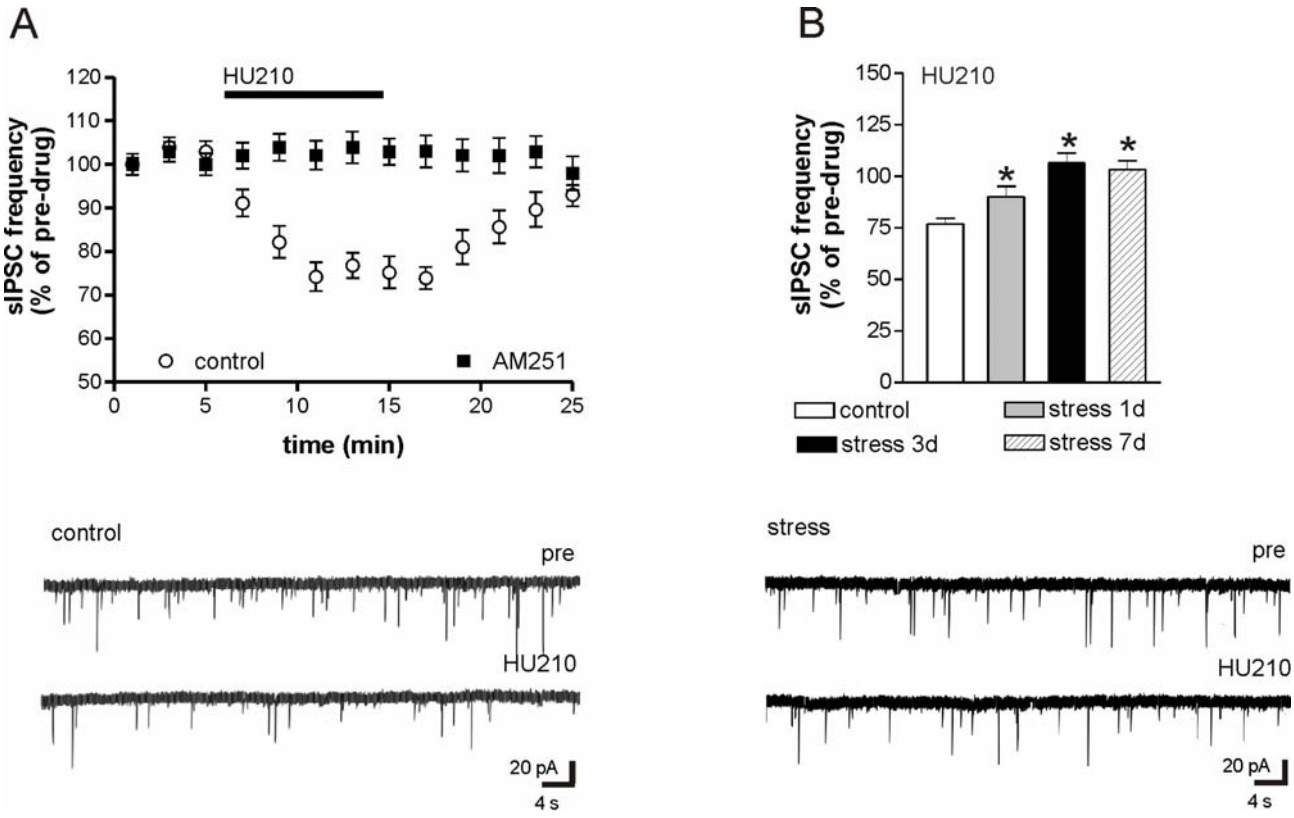

Figure 1. Stress alters the sensitivity of striatal GABA synapses to the stimulation of cannabinoid CB1 receptors. A. HU210, agonist of CB1 receptors, reduced sIPSC frequency in control mice. This effect was fully prevented by preincubation with the CB1 receptor antagonist AM251. B. The graph shows that the depressant effect of HU210 on sIPSC was attenuated in striatal mice exposed to 1 session of stress, and it was completely abolished in neurons from mice exposed to stress for 3 and 7 consecutive days. The electrophysiological traces below are examples of voltage-clamp recordings showing that HU210 (10 min) failed to reduce mIPSC frequency in a mouse exposed to 3 days of stress. *means <0.05 compared to control mice

Conversely, in neurons from mice exposed to rewarding paradigms (7 and 15 days for sucrose-containing solution; 15 and 30 days for running wheel), HU210 effects were remarkably potentiated (n= at least 14, $p<0.01$ compared to controls for each time point and both experimental groups), indicating increased sensitivity of GABA synapses to cannabinoid receptor stimulation (Fig. 2A, B). In these mice, basal sIPSC frequency (stress (3 days): 1.37±0.06 Hz; stress (7 days): 1.32±0.05 Hz; sucrose (7days):1.38±0.05 Hz; sucrose (15 days): 1.41±0.06 Hz; wheel (15 days): 1.38±0.05 Hz; wheel (30 days): 1.37±0.04 Hz; control: 1.40±0.04 Hz) and amplitude (stress (3 days): 31.6±1.5 pA; stress (7 days): 32.1±1.7 pA; sucrose (7days): 33.3±1.5 pA; sucrose (15 days): 31.1±1.6 pA; wheel (15 days): 38.2±1.5 pA; wheel (30 days): 37.1±1.4 pA; control: 30.8±1.4 pA) were normal (n= at least 10 and $p>0.05$ for each experimental and control group).

Notably striatal neurons of mice exposed to one day of social defeat (n=13), HU210 effects were still present although they were significantly attenuated ($p<0.05$; Fig. 1B), whereas in striatal neurons from mice exposed to sucrose-containing solution for 1 and 3 days or to running wheel for 1, 3 and 7 days, HU210 produced similar effects to their respective controls (n=at least 8, $p>0.05$ respect to controls for each time point; Fig 2C,D).

In striatal neurons from control mice, as well as from mice exposed to sucrose-containing solution (7 days) or to running wheel (15 days), HU210 effects were prevented by pre-incubating (5-10 min) the slices with AM251, antagonist of CB1 receptors (n=6 and $p>0.05$ for each group) (Fig. 2C, D). AM251 was unable to alter *per se* the frequency of sIPSCs in control mice, as well as in mice exposed to sucrose-containing solution (7 days) or to running wheel (15 days), and in stressed (1, 3 and 7 days) mice (n=at least 7 cells and $p>0.05$ for each experimental group) (not shown).

To isolate synaptic GABA events from the possible contamination of pre- and postsynaptic spiking activity, miniature GABAergic currents (mIPSCs) were recorded following the application of TTX (5-10 min), a selective voltage-dependent sodium channel blocker. As described, mean frequency of mIPSCs was significantly lower compared to sIPSCs recorded from control neurons and from neurons exposed to sucrose-containing solution (7 days) or to running wheel (15 days), and in

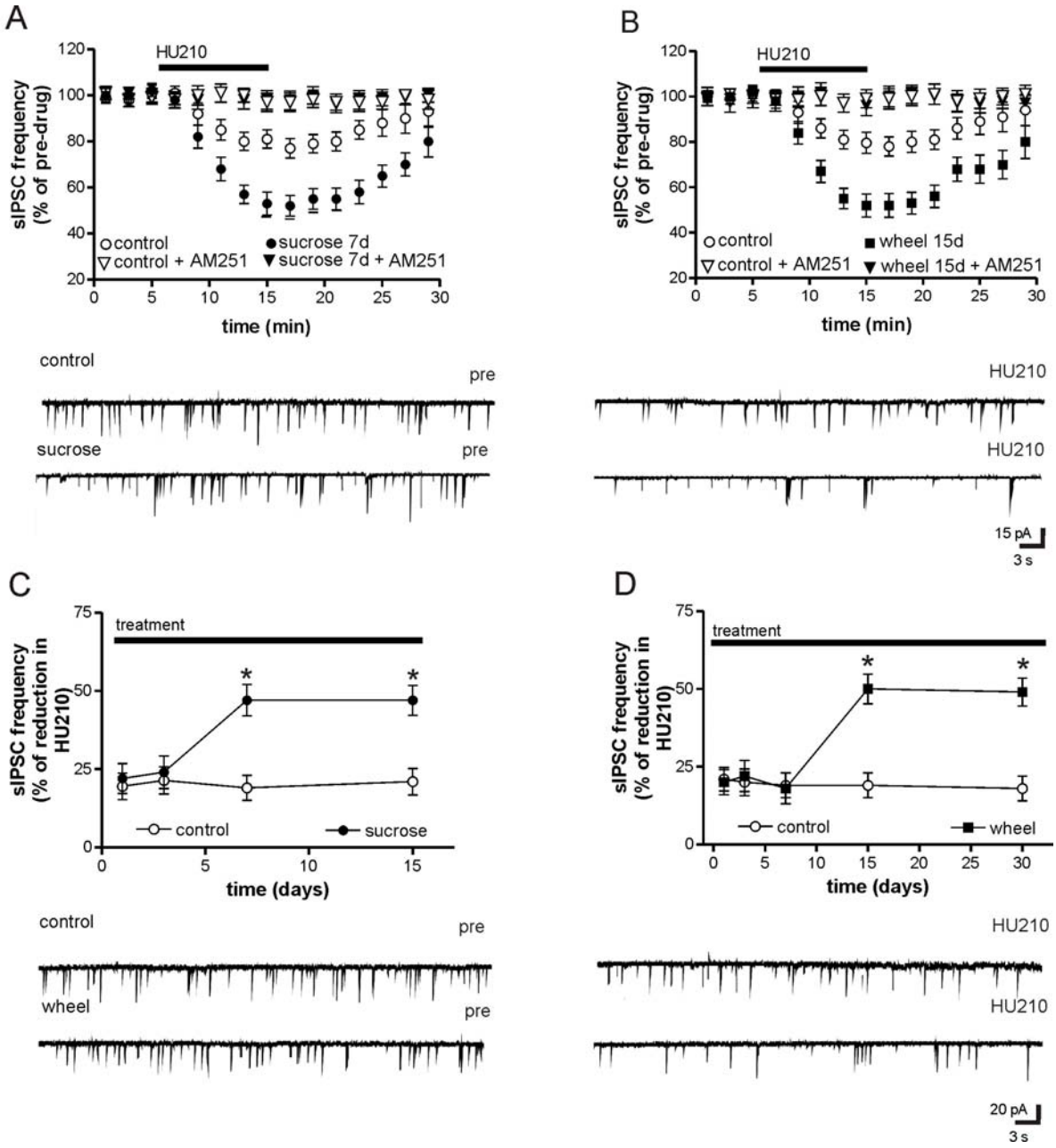

Figure 2. Running wheel and sucrose consumption potentiate the effects of HU210 on striatal sIPSCs. A. The graph shows the effects of HU210 on sIPSCs were potentiated after 7 days of sucrose exposure. Preincubation with the CB1 receptor antagonist AM251 prevented the depressant action of HU210 both in control mice and in sucrose-exposed mice. B. The graph shows the effects of HU210 on sIPSCs were potentiated after 15 days of wheel running. Preincubation with the CB1 receptor antagonist AM251 prevented the depressant action of HU210 both in control mice and in wheel-exposed mice. C. The graph shows that HU210-induced reduction of sIPSC frequency was normal after 1 and 3 days of exposure to sucrose-containing solution. Conversely , the effects of HU210 on sIPSCs were potentiated after 7 and 15 days of sucrose exposure. D. The graph shows that HU210-induced reduction of sIPSC frequency was normal after 1,3 and 7 days of exposure to running wheel. Conversely, the effects of HU210 on sIPSCs were potentiated after 15 and 30 days of exposure to running wheel. The electrophysiological traces on the bottom are examples of voltage-clamp recordings before and during the application of HU210 in control sucrose (7 days)- and running wheel (15 days)-exposed mice. * means $p<0.05$ compared to control mice.

stressed (1, 3 and 7 days) mice (n=at least 12 and $p<0.05$ respect to pre-drug values for each experimental group), Furthermore, no differences were evident between mIPSC frequencies and amplitudes recorded in control, stressed, running wheel- or sucrose-receiving animals (n=at least 12, $p>0.05$ for both parameters) (not shown).

We investigated the electrophysiological effects of HU210 on mIPSCs recorded from control mice and in mice exposed to running wheel (15 days), to sucrose (7 days) or to stress (3 days).

In slices from mice exposed to running wheel and to sucrose, the inhibitory effects of HU210 on mIPSC frequency were significantly greater (n=at least 14 $p<0.01$ respect to controls) (Fig. 3A, B). Coversely HU210 was also ineffective in reducing the frequency of mIPSCs in neurons from stressed (3 days) mice (n=9 $p>0.05$; Fig. 3C).

Spontaneous IPSC and mIPSCs were fully blocked by bicuculline in control mice, as well as in mice with access to running wheel (15 days), to sucrose-containing drinking solution (7 days), and exposed to stress (3 days) (n= at least 8 for the three synaptic parameters and for each experimental groups), indicating that these synaptic events were entirely mediated in the three experimental groups by the stimulation of GABAA receptors (not shown).

The effects of social defeat and natural rewards paradigms or were slowly reversible after the discontinuation of both treatments, so that the sensitivity of sIPSCs to HU210 returned to control values 3 and 7 days after the last of 3 consecutive sessions (1 per day) of social stress, 3 days of sucrose discontinuation (n=6, $p>0.05$ compared to controls) or after 7 days of running wheel discontinuation (n=7, $p>0.05$) (Fig. 3D-F).

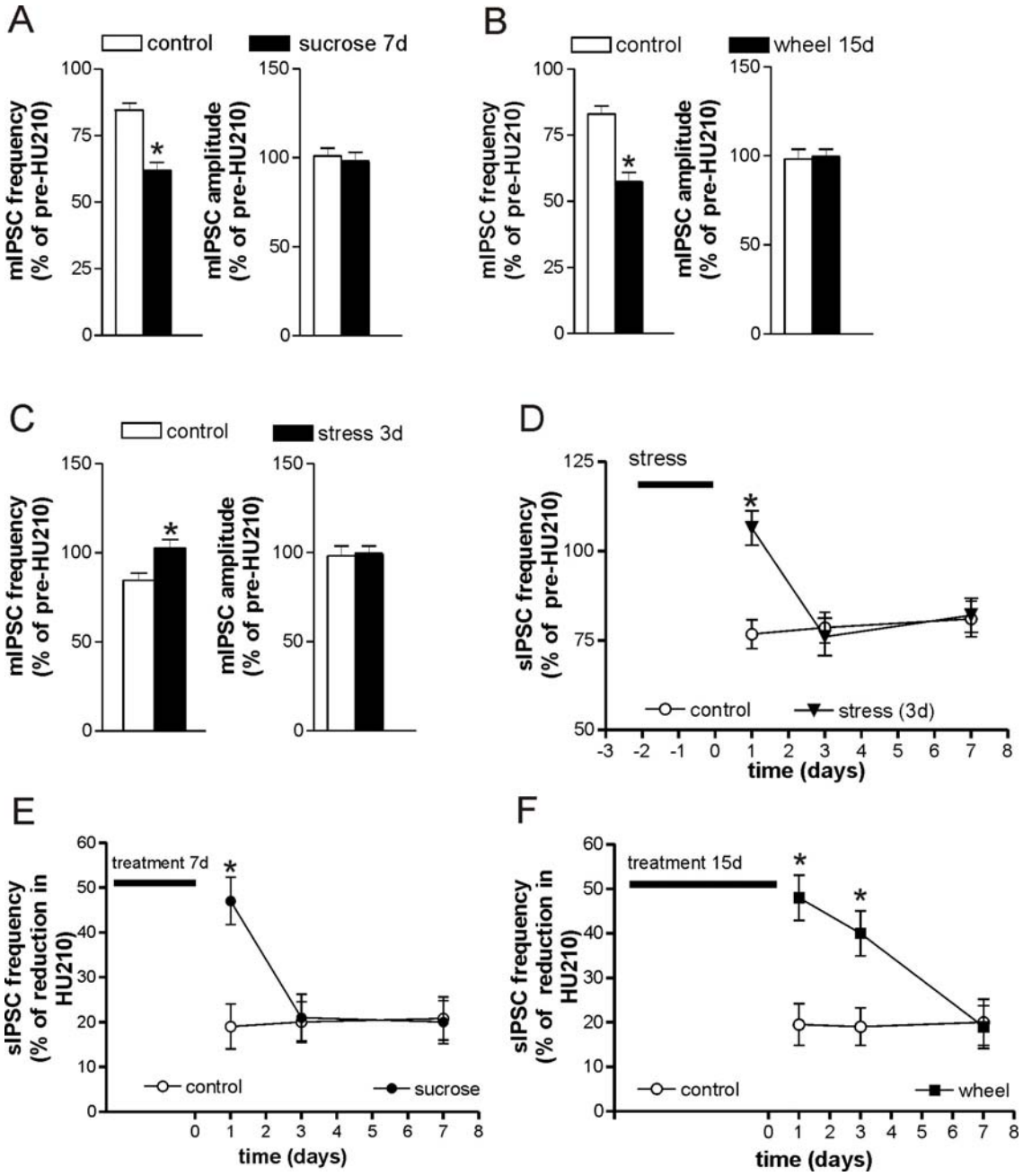

Figure 3. Running wheel, sucrose and stress exposure modify the effects of HU210 through a presynaptic action. A,B. The activation of CB1 receptors with HU210 reduced mIPSC frequency but not amplitude in control and in rewarded mice. In sucrose (7 days)-receiving mice (A) and in wheel (15 days)-exposed mice (B) the effect of HU210 was potentiated. C. The graph shows that the depressant effect of HU210 on mIPSC was completely abolished in neurons from mice exposed to stress for 3 consecutive days. HU210 does not alter mIPSC amplitude.

Effects of time on stress- and natural rewards-induced alteration of HU210 responses. D. The graphs show that the reduction of sIPSC frequency induced by HU210 in mice exposed to stress for 3 days was lost 3 and 7 days after the last stress session. E. The graph shows that the depressant effects of HU210 on sIPSC frequency were still potentiated in mice exposed for 7 days to sucrose-containing solution and recorded 1 day after. In mice recorded 3 and 7 days after the last exposure to sucrose, HU210 produced normal effects. F. The depressant effects of HU210 on sIPSC frequency were still potentiated in mice exposed to running wheel for 15 days and recorded 1 and 3 days after. In mice recorded 7 days after the last exposure to running wheel, HU210 produced normal effects. * means $p<0.05$ compared to control mice.

Effects of HU210 on striatal glutamate transmission

In the striatum, stimulation of cannabinoid CB1 receptors presynaptically reduces glutamatergic transmission (Gerdeman and Lovinger, 2001; Huang *et al*, 2001; Centonze *et al*, 2005). Thus, to see whether the altered response to CB1 receptor stimulation found in mice exposed to running wheel, to sucrose and to stress was restricted to GABA-mediated sIPSCs or also involved glutamate transmission, we tested the effects of HU210 on glutamate-mediated sEPSCs. Frequency and amplitude of sEPSCs were not significantly different in control condition and after exposure to running wheel (15 days), to sucrose (7 days) and to stress (3days) ($n=9$ and $p>0.05$ for both parameters and each group). HU210 reduced the frequency of sEPSCs in all the tested neurons ($n=12$ and $p<0.05$ respect to pre-drug values for each group), and produced remarkably similar effects in control mice and after running wheel, sucrose and stress exposure ($p>0.05$ compared to controls) (Fig. 4A-C).

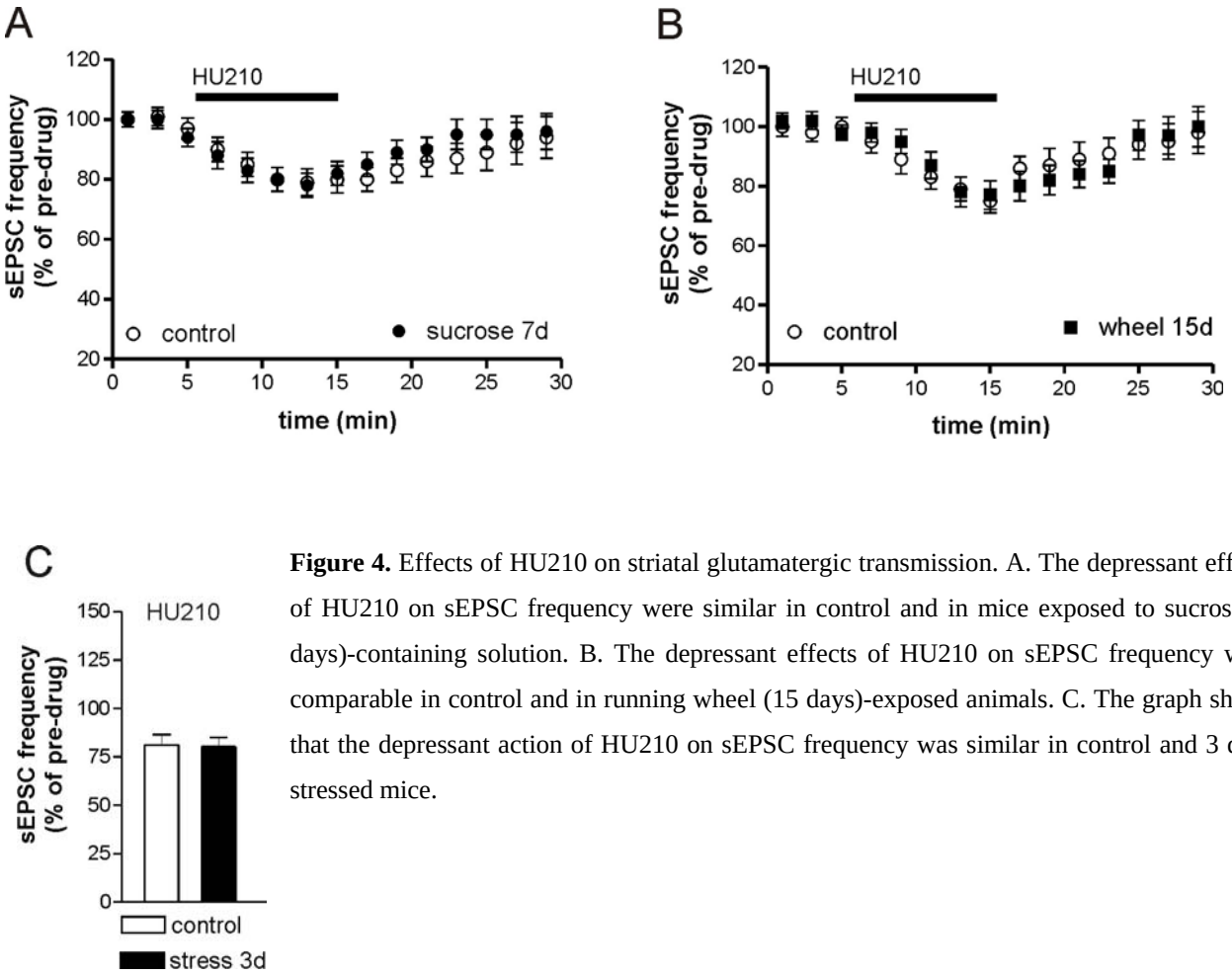

Figure 4. Effects of HU210 on striatal glutamatergic transmission. A. The depressant effects of HU210 on sEPSC frequency were similar in control and in mice exposed to sucrose (7 days)-containing solution. B. The depressant effects of HU210 on sEPSC frequency were comparable in control and in running wheel (15 days)-exposed animals. C. The graph shows that the depressant action of HU210 on sEPSC frequency was similar in control and 3 days stressed mice.

Effects of baclofen on GABA transmission after running wheel, sucrose consumption and stress

Many receptors participate in the presynaptic modulation of GABA transmission in the striatum, including the GABAB receptors (Calabresi *et al*, 1991). Thus, we investigated whether the abnormal control of GABA synapses observed in rewarded and stressed mice was specific for the CB1 receptors or also involved other presynaptic receptors. Application of the GABAB receptor agonist baclofen (10 min) reduced striatal sIPSC frequency in control mice (n=8 and $p<0.01$). In neurons from mice exposed to sucrose (7 days), to running wheel (15 days) or to stress (3 days) the effects of baclofen were similar to those observed in control animals (n=at least 8 for each experimental group, $p>0.05$ compared to baclofen in non-rewarded mice) (Fig. 5A-C).

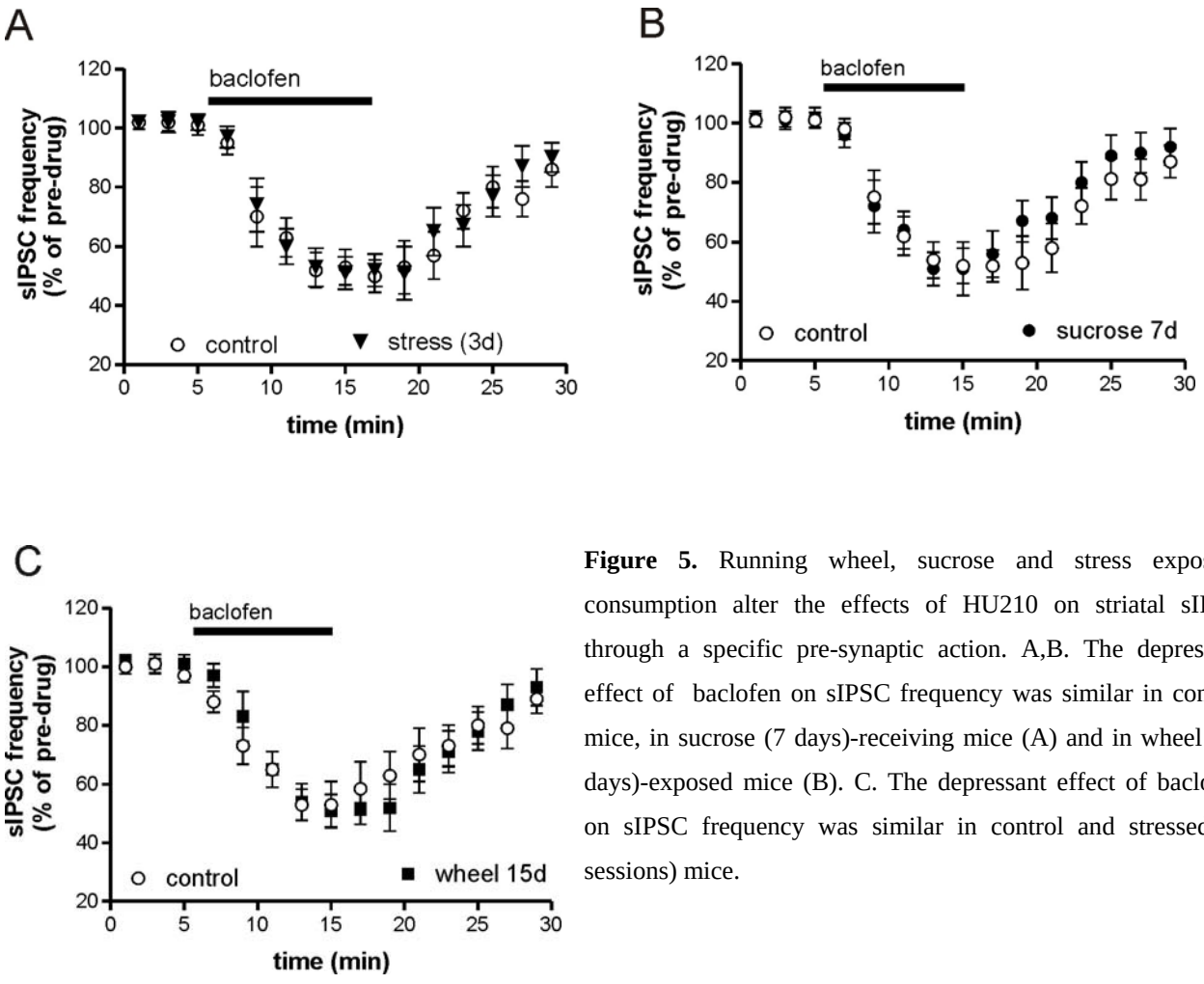

Figure 5. Running wheel, sucrose and stress exposure consumption alter the effects of HU210 on striatal sIPSC through a specific pre-synaptic action. A,B. The depressant effect of baclofen on sIPSC frequency was similar in control mice, in sucrose (7 days)-receiving mice (A) and in wheel (15 days)-exposed mice (B). C. The depressant effect of baclofen on sIPSC frequency was similar in control and stressed (3 sessions) mice.

Effects of DHPG on striatal mIPSCs following running wheel or sucrose

It has been recently reported that activation of metabotropic glutamate receptors 5 by 3,5-DHPG mobilizes endocannabinoids in the striatum (Jung *et al*., 2005; Maccarrone *et al*., 2008), and that this effect results in the inhibition of GABA-mediated sIPSCs and of mIPSCs through the stimulation of cannabinoid CB1 receptors (Centonze *et al*., 2007b; Maccarrone *et al*., 2008. Application of 3,5-DHPG (30 min, n=18) caused a biphasic effect on sIPSCs in control mice, since it initially increased ($p<0.01$ at 5 min), and then reduced the frequency of these events ($p<0.05$ at 20 min). Both mGlu 1 and 5 receptors were involved in the early response to 3,5-DHPG, through membrane depolarization of striatal GABAergic interneurons and action potential generation. The 3,5-DHPG-mediated late depression of inhibitory inputs to striatal principal neurons was conversely secondary to mGlu 5 receptor activation and subsequent endocannabinoid release and was prevented by the antagonist of the cannabinoid CB1 receptor AM251 (n=7) (Centonze *et al*., 2007b).

Thus, to see whether stress altered the sensitivity of GABA synapses not only to the synthetic cannabinoid HU210 but also to endocannabinoids, we measured the effects of 3,5-DHPG on striatal sIPSCs recorded from mice exposed to 3 sessions of stress. According to the idea that social stress altered the sensitivity of striatal GABA synapses also to endocannabinoids, we observed that 3,5-DHPG produced only a sustained increase of sIPSC frequency in stressed mice (n=13, $p<0.05$ at 5, 10 and 20 min), since the late sIPSC inhibition was absent (Fig. 6).

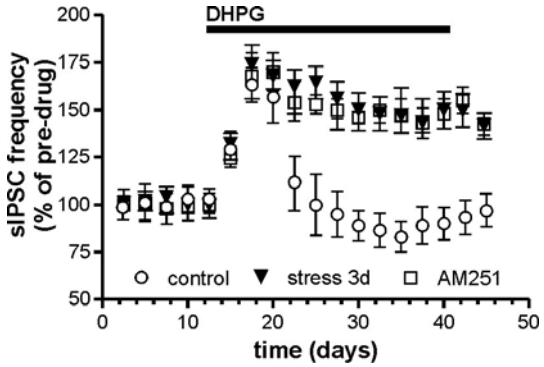

Figure 6. Effects of 3,5-DHPG on GABA transmission in stressed mice. The graph shows the early increase and the late reduction of sIPSC frequency after the application of a group I mGlu receptor agonist 3,5-DHPG in control mice. Preincubation with AM251 or 3 sessions of stress prevented the late endocannabinoid-dependent inhibition of sIPSCs.

In order to better appreciate the effects of running wheel 15 days and sucrose 7 days on the sensitivity of GABA synapses to 3,5-DHPG, mIPSCs were recorded following the application of TTX, to block the early response due to the action potential generation and to isolate the late endocannabinoid-dependent depression of inhibitory inputs to striatal principal neurons.

Application of 3,5-DHPG (10 min, n=10) significantly inhibited mIPSC frequency in control mice ($p<0.05$ respect to pre-drug value), and produced a greater effect after running wheel or sucrose consumption (n=at least 11 and $p<0.05$ compared to controls for the two experimental groups) (Fig. 7A,C). AM251 fully blocked the effects of 3,5-DHPG in control mice and in mice exposed to running wheel and to sucrose (n=4 and $p>0.05$ for each group) (Fig. 7B,D).

Role of corticosteroids on stress-induced inhibition of HU210 responses

Many effects of stress in the nervous system are secondary to the activation of the hypothalamic-pituitary-adrenal axis and to the subsequent increase in plasma concentrations of corticosteroids (Piazza and Le Moal, 1998; Chrousos and Kino, 2007). To see whether the altered response to cannabinoid receptor stimulation seen in defeated mice was caused by corticosteroids, we pre-treated mice with i.p. injections of RU486 (n=8), antagonist of glucocorticoid receptors, or with vehicle (n=8), 5-10 min before exposure to each session of the stress protocol (3 consecutive days). RU486 did not alter basal sIPSC frequency (1.29±0.05 Hz versus 1.33±0.05 Hz, $p>0.05$) and amplitude (30.6±1.9 pA versus 31.5±1.3 pA, $p>0.05$), but it was able to prevent the effects of stress on HU210 responses (n=16, $p<0.01$), while i.p. vehicle did not (n=16, $p>0.05$).

In another set of experiments, unstressed mice (n=6) were administrated subcutaneously with corticosterone once a day for 3 consecutive days. A similar corticosterone injection paradigm has been reported to cause a persistent elevation of plasma corticosterone lasting for about 24 h (Sousa *et al.*, 1998). In these mice, sIPSC frequency (1.30±0.04 Hz versus 1.32±0.05 Hz, $p>0.05$) and amplitude (34.3±1.5 pA versus 31.9±1.2 pA, $p>0.05$) were normal, but HU210 was ineffective in reducing GABA transmission ($p>0.05$, n=14), as described for stressed mice (Fig. 8).

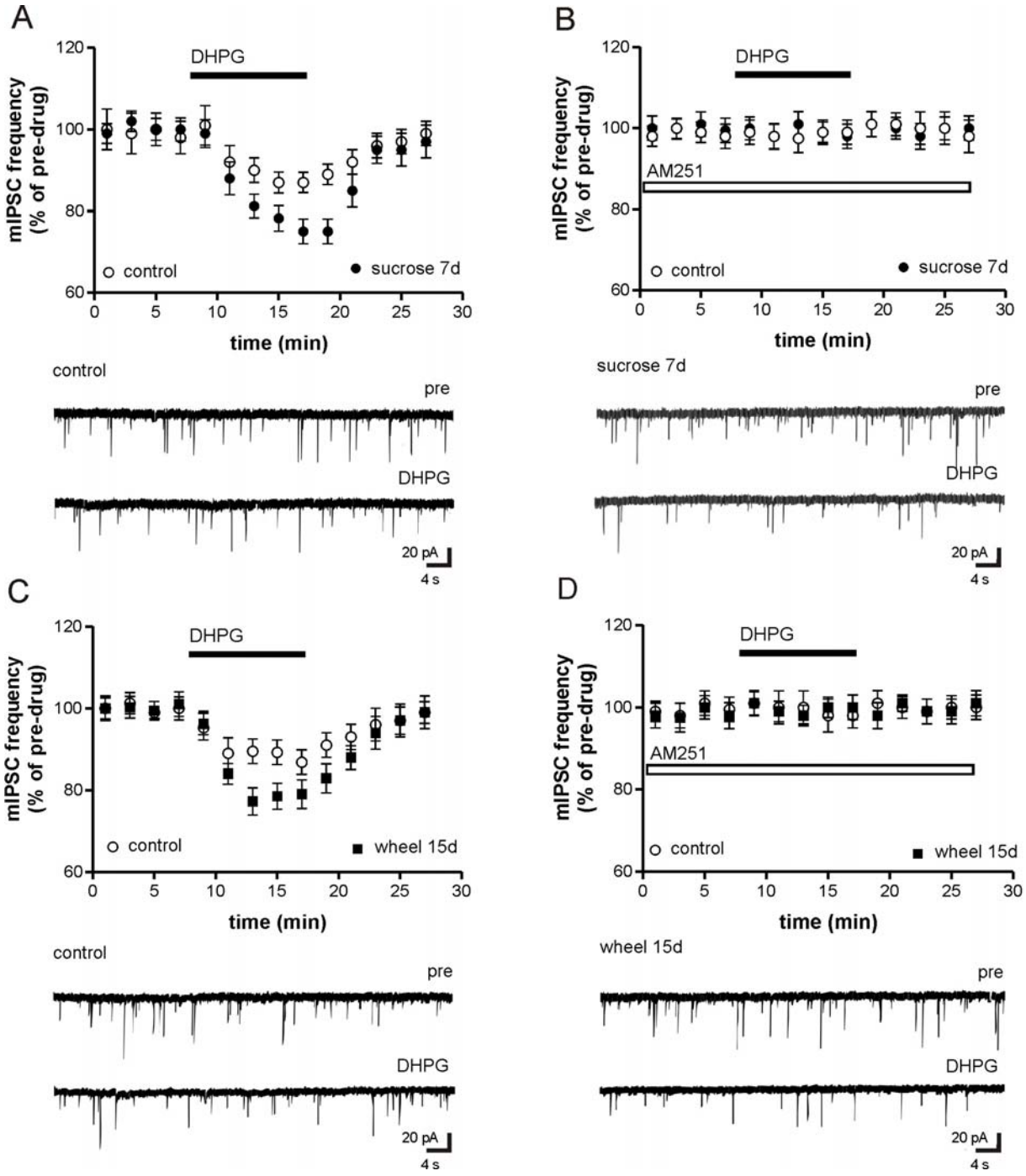

Figure 7. Effects of 3,5-DHPG on striatal mIPSCs. A. The reduction of mIPSC frequency after the application of the group I mGlu receptor agonist 3,5-DHPG was potentiated in sucrose (7 days)-treated mice. B. Preincubation with the CB1 receptor antagonist AM251 prevented the depressant action of 3,5-DHPG in control and sucrose (7 days)-receiving mice. Traces on the bottom are voltage-clamp recordings before and during the application of 3,5-DHPG in control and sucrose (7 days)-treated mice. C. The graph shows that the reduction of mIPSC frequency after the application of 3,5-DHPG was increased by running wheel (15 days). D. Preincubation with AM251 prevented the depressant action of 3,5-DHPG in control and in running wheel (15 days)-exposed mice. The traces on the bottom are examples of voltage-clamp recordings before and during the application of 3,5- DHPG in control and in running wheel (15 days)-exposed mice.

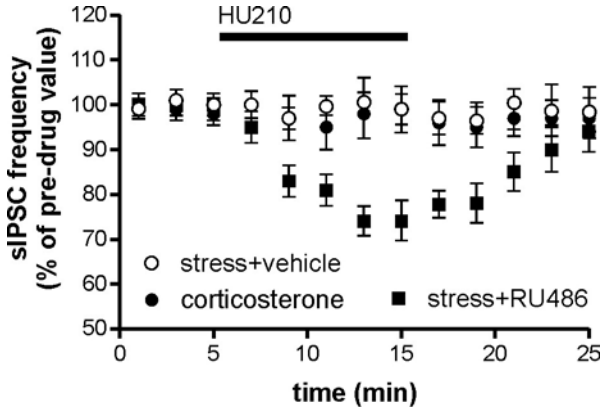

Figure 8. Blockade of glucocorticoid receptors, natural rewards and cocaine reverse the inhibition of HU210 responses induced by stress. The graph shows that pretreatment with RU486, antagonist of glucocorticoid receptors, prevented the effects of 3 sessions of stress on HU210-induced reduction of sIPSC frequency and that corticosterone treatment mimicked the stress-induced effects.

Role of natural reward in protecting from and reverting the effects of stress

In this study we have described that the anxious state induced by chronic psychoemotional stress is associated with the loss of cannabinoid CB1-mediated control of striatal sIPSCs. Thus, we first assessed whether running wheel- or sucrose-induced sensitization of cannabinoid CB1 receptors was associated with behavioral changes. Neither running wheel (15 days) nor sucrose (7 days) produced *per se* overt anti-anxiety effects, as revealed at the open-field protocol (not shown). We next investigated whether the sensitization of cannabinoid responses after both environmental manipulations may protect from both the emotional and synaptic consequences of stress. Anxiety of mice was revealed by their motor behavior in an aversive open-field environment. We studied locomotor activity of mice daily exposed to a stressing aggressor or given access for 15 days to running wheel or for 7 days to sucrose before stress induction, and compared their motor responses to that of control standard-housed animals. Analysis of total distance traveled by one-way ANOVA displayed a significant effect of treatment ($F_{3,41}$=3.641, p=0.0204). Following *post-hoc* comparison (Student's *t*-test) revealed that mice exposed to social stress were significantly less active than control littermates (p=0.0061). In contrast, animals exposed to running wheel (15 days) or to sucrose solution (7 days) before daily aggressions displayed a locomotor activity comparable to that of control mice (running wheel, p=0.8915; sucrose, p=0.8164) (Fig. 9A). Further dissection of horizontal locomotion, measured in time intervals, confirmed a dramatic stress-induced hypoactive phenotype of mice exposed to aggressor (two-way ANOVA: treatment effect, $F_{1,46}$=7.602,

p=0.0112; treatment x time interaction, $F_{2,46}$=5.416, p=0.0077) (Fig. 9B). In contrast, motor activity profile was normal in pre-rewarded animals (running wheel, treatment effect, $F_{1,42}$=0.012, *p*=0.9124, treatment x time interaction, $F_{2,42}$=0.539, *p*=0.5874; sucrose, treatment effect, $F_{1,42}$=0.034, *p*=0.8563, treatment x time interaction, $F_{2,42}$=1.312, *p*=0.2802). In the open-field test, center/total distance ratio can be used as an index of anxiety-related responses, since anxiety reduces the time spent in the center of the arena. Analysis of center/total distance ratio revealed a significant increase of anxiety in mice exposed to stress since they spent a shorter time in the center of the arena compared to standard-housed mice (two-way ANOVA: treatment effect, $F_{1,46}$=13.249, p=0.0014; treatment x time interaction, $F_{2,46}$=2.086, p=0.1358) (Fig. 9C). Differently, cage enrichment with running wheel or sucrose were both able to completely reverse the anxiogenic effect of social defeat stress (running wheel: treatment effect, $F_{1,42}$=0.248, p=0.6238, treatment x time interaction, $F_{2,42}$=0.055, p=0.9464; sucrose: treatment effect, $F_{1,42}$=1.960, p=0.1761, treatment x time interaction, $F_{2,42}$=0.923, p=0.4053).

Consistently with the behavioral data described above, both sucrose (7 days) and running wheel (15 days) also protected from the synaptic consequences of stress, since HU210 did not reduce sIPSC frequency in stressed animals (n=8, p>0.05 compared to pre-HU210 values), but was able to inhibit these GABA-mediated synaptic events after three days of stress in mice previously exposed to the running wheel (n=9, p<0.01 compared to pre-HU210 values) or to the sucrose-containing drinking solution (n=11, p<0.01 compared to pre-HU210 values) (Fig. 9D).

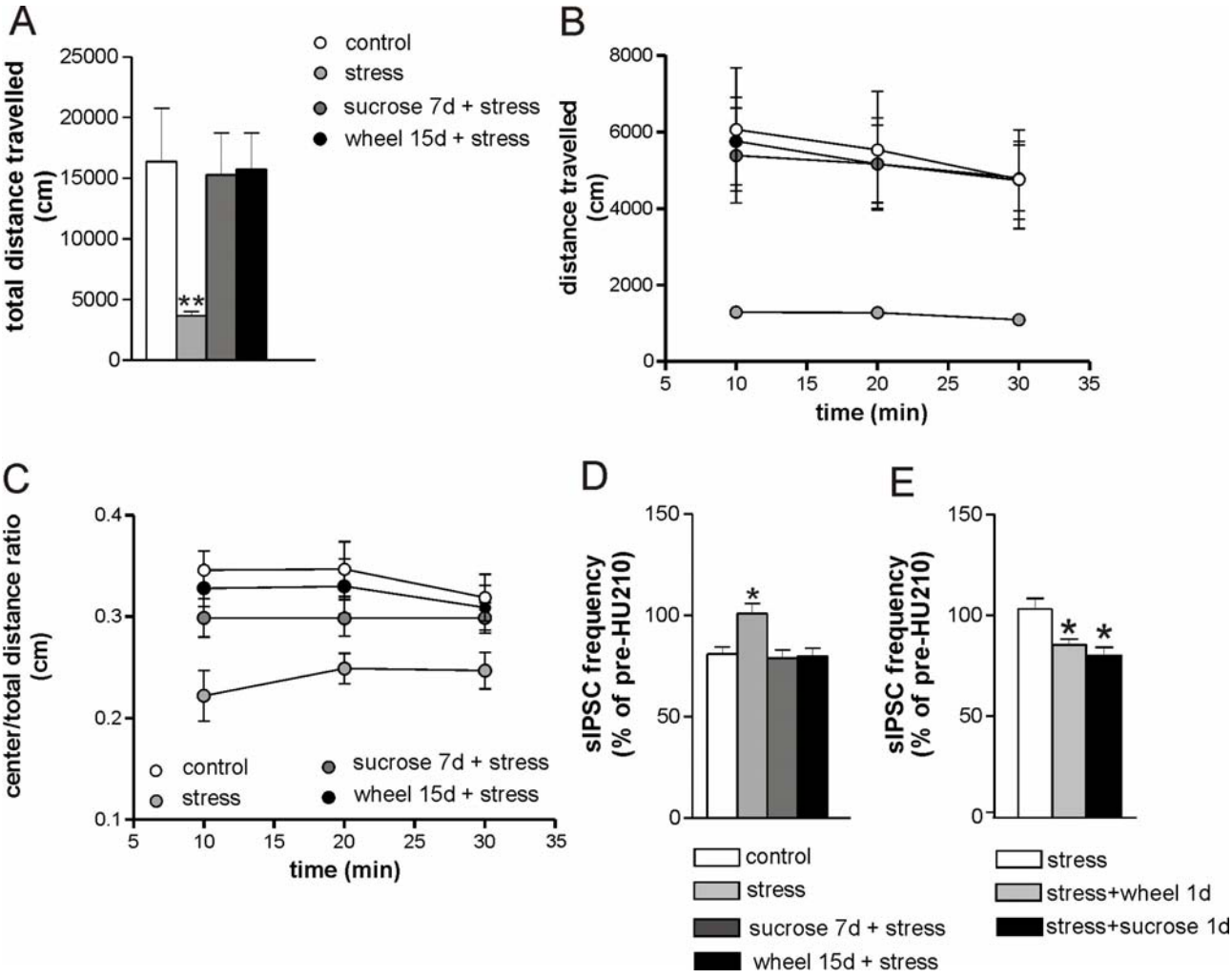

Figure 9. Running wheel and sucrose consumption protect mice from the behavioral effect of social defeat stress. Mice were allowed to explore an open-field box for 30 min. A. Total distance travelled by mice subjected to social stress was significantly reduced compared to non-stressed animals. On the contrary, mice previously exposed to 15 days of running wheel or to 7 days of sucrose consumption showed a locomotor activity comparable to controls. B. Similar results were obtained by recording the profile of locomotion over successive 10-min intervals. C. The distance covered in the centre compared to the total distance was used as a parameter of anxiety-related behavior. In accordance to motor activity, this index of emotionality revealed that running wheel and sucrose rewards are able to prevent stress-induced anxiety. Indeed, mice exposed to natural rewards before stress displayed distance ratios comparable to those of their control non-stressed littermates. D. The graph shows that natural rewards were able to revert the synaptic consequences of stress: sucrose (7 days)-treatment and running wheel (15 days)-exposure were able to rescue the effect of HU210 on sIPSC frequency. E. The graph shows that natural rewards like wheel running or sucrose drinking were able to rescue the effect of HU210 on sIPSC frequency in mice exposed to 3 days of stress. *means $p<0.05$ compared to control mice. ** means $p<0.01$ compared to control mice.

We also investigated whether enriched environment with rewarding properties might be able to reverse the effects of stressful events on cannabinoid transmission. In a further set of experiments, stressed mice (3 days n=8) were introduced for 24 h in a novel cage containing a running wheel immediately after the completion of the social defeat protocol. The sensitivity of striatal sIPSCs to HU210 was normal in these mice (n=18, $p<0.01$), indicating a rescue of the effects of stress. Similar results were obtained in stressed mice (3 days, n=6) which were allowed, immediately after the third stress session, to consume *ad libitum* a drinking fluid containing sucrose for 24 h. Also in these animals, in fact, the effect of HU210 on GABAergic sIPSC frequency was normal (n=14, $p<0.01$) (Fig. 9E). Notably, neither 24 h running wheel nor 24 h sucrose produced significant effects on HU210-induced sIPSC inhibition when administered in non-stressed mice (Fig. 1E,F).

BDNF blocks CB1 receptors effects on striatal GABA synapses via tyrosine kinase activation

We first tested the effects of *in vitro* BDNF on CB1 receptors on striatal GABA synapses function. Incubation of striatal slices with BDNF did not alter sIPSC frequency (control: 1.33 ± 0.38 Hz, BDNF: 1.25 ± 0.23 Hz; $p>0.05$; n=8 for both groups) or amplitude (control: 33.20 ± 3.28 pA, BDNF: 31.70 ± 3.97 pA; $p>0.05$; n=8 for both groups), but fully blocked the effects of the CB1 receptors agonist HU210 on sIPSC frequency. In BDNF-treated slices, HU210 effects were negligible on both sIPSC frequency and amplitude (n=8; $p>0.05$; Fig. 10A).

The effects of BDNF are largely mediated by the TrkB tyrosine kinase receptor, and previous studies in slices reported that they were prevented by inhibiting tyrosine kinases with lavendustin A (Frerking *et al*., 1998). Lavendustin A was unable *per se* in altering sIPSC frequency or amplitude (n=6; $p>0.05$ for both parameters), and it did not affect HU210 responses (n=6; $p>0.05$ compared to HU210 alone). This pharmacological compound, however, fully prevented the effects of BDNF on HU210-mediated inhibition of sIPSC frequency (n=7; $p<0.01$; Fig. 10B,C).

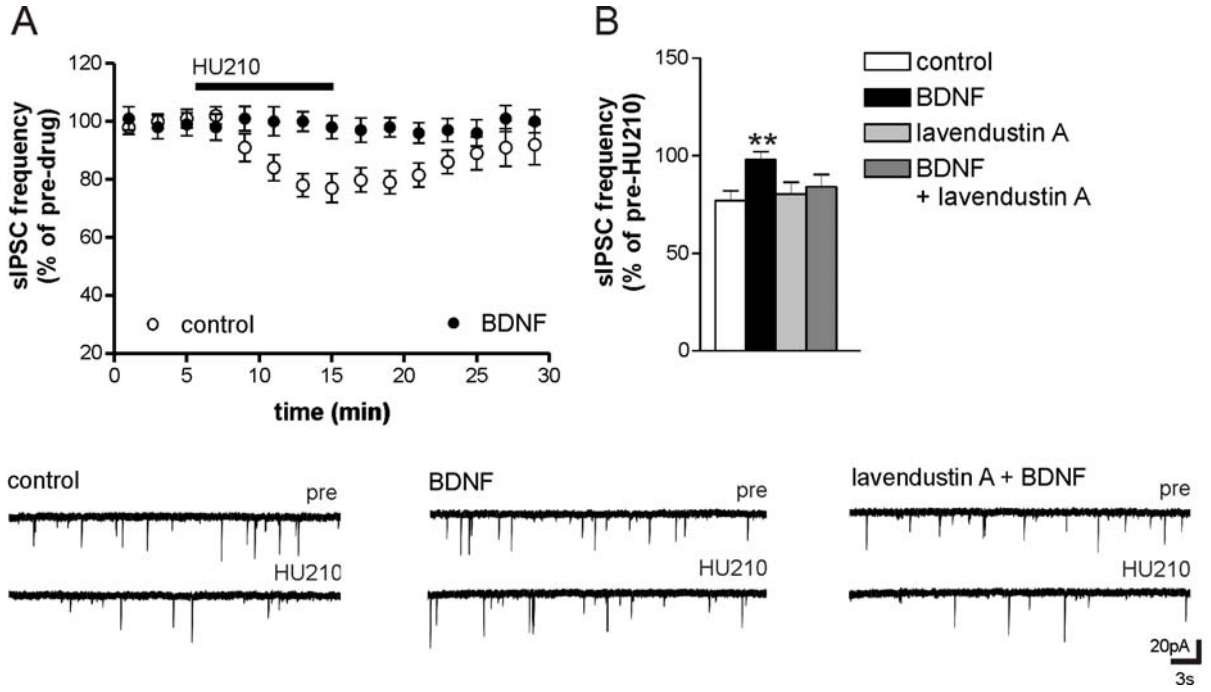

Figure 1. BDNF blocks CB1 receptor function on striatal GABA synapses via tyrosine kinase activation. A. The graph shows that the depressant effect of the CB1 receptor agonist HU210 was completely abolished by incubation of striatal slices with BDNF. B. Lavendustin A, an inhibitor of TrkB tyrosine kinase, did not affect *per se* HU210 responses on sIPSC frequency, but was able to rescue the effect of HU210 in BDNF-treated slices. The electrophysiological traces below are examples of voltage clamp recordings showing the loss of mIPSC frequency reduction induced by HU210 in a neuron from BDNF-treated slices and the recovery of the effects of HU210 induced by lavendustine A. **, $p<0.01$ compared to control mice.

GABAB receptors and CB1 receptors on striatal glutamate synapses are insensitive to BDNF

Striatal GABA synapses are modulated by many receptor subtypes inhibiting transmitter release. Thus, we investigated whether the effect of BDNF on presynaptic control of GABA transmission was specific for CB1 receptors on GABA synapses or also involved other receptors. Application of the GABAB receptor agonist baclofen (n=7) significantly ($p<0.01$) reduced striatal sIPSC frequency in control slices (Calabresi *et al*., 1991). In BDNF-treated slices, the inhibitory effect of baclofen was similar to that seen in control condition (n=8, $p>0.05$ compared to baclofen in control slices; Fig. 11A).

CB1 receptors also control glutamate transmission in the striatum by a presynaptic mechanism. According to previous findings (Maccarrone *et al*., 2008), HU210 inhibited glutamate-mediated sEPSC frequency in control slices (n=6; $p<0.01$). In BDNF-treated slices, the effect of HU210 on

sEPSCs was indistinguishable from that of controls (n=7, p>0.05 compared to HU210 in control slices), indicating that BDNF selectively modulates CB1 receptors regulating GABA synapses (Fig. 11B).

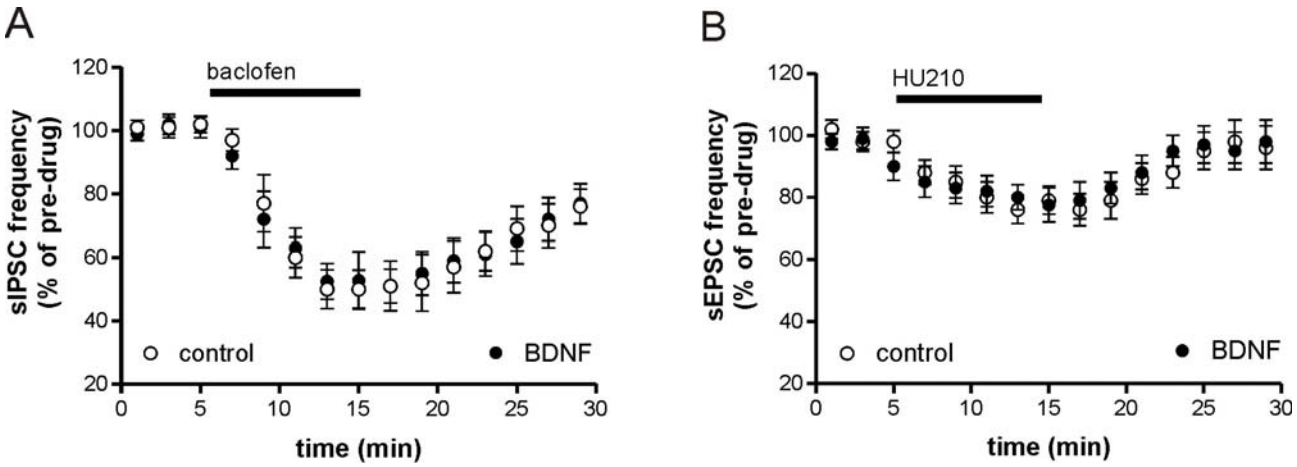

Figure 11. GABAB receptors and CB1 receptors on glutamate synapses are insensitive to BDNF. A. The depressant effect of the GABAB receptor agonist baclofen on sIPSC frequency was similar in control and BDNF-treated slices. B. The graph shows that the depressant action of HU210 on sEPSC frequency was similar in control and BDNF-treated slices.

***In vivo* manipulations of BDNF levels alter the sensitivity of CB1 receptors on GABA synapses**

In a further set of experiments, we wanted to explore whether alterations of BDNF levels in living animals resulted in concomitant changes of CB1 receptor sensitivity in the striatal GABA synapses. BDNF administered *via* a single i.c.v. injection resulted in the complete suppression of HU210 effect on sIPSC frequency (n=6 for both i.c.v. BDNF and i.c.v. vehicle; p>0.05 for BDNF and p<0.01 for vehicle; Fig. 12A). Both i.c.v. BDNF and i.c.v. vehicle failed to affect sIPSC frequency and amplitude (not shown).

We also measured the effects of HU210 on sIPSCs in mice with partial genetic BDNF depletion (Jeanblanc *et al*., 2006; Saylor and McGinty, 2008). In BDNF+/- mice, sIPSC frequency (1.14 ± 0.25 Hz) and amplitude (32.81 ± 4.10 pA) were similar to those of the respective control group (sIPSC frequency: 1.27 ± 0.32 Hz; sIPSC amplitude: 35.17 ± 3.82 pA) (n=6; p>0.05 for both parameters; not shown). Conversely, HU210-mediated inhibition of sIPSC frequency was

selectively potentiated in striatal neurons from BDNF+/- mice (n=6; $p<0.05$ compared to HU210 in control mice). Even in these mice, AM251 prevented the effect of HU210 on sIPSCs, confirming that BDNF plays a crucial role in the control of striatal GABAergic CB1 receptors (Fig. 12B-D).

BDNF is able to fully block sensitized CB1 receptors on GABA synapses

To see whether BDNF was able to contrast the activity of striatal CB1 receptors on sIPSC not only in normal condition, but also after natural reward paradigms, we have administered BDNF i.c.v. after exposure to running wheel (15 days), to sucrose (7 days). BDNF fully blocked the HU210-mediated inhibition of sIPSCs (n=6; $p>0.05$ for both running wheel and sucrose), while i.c.v. vehicle did not prevent the enhancing effects of both running wheel (n=6) and sucrose drinking (n=6) on HU210-mediated inhibition of sIPSC frequency (n=6) ($p<0.01$ compared to i.c.v. vehicle administered in non rewarded animals; Fig. 13).

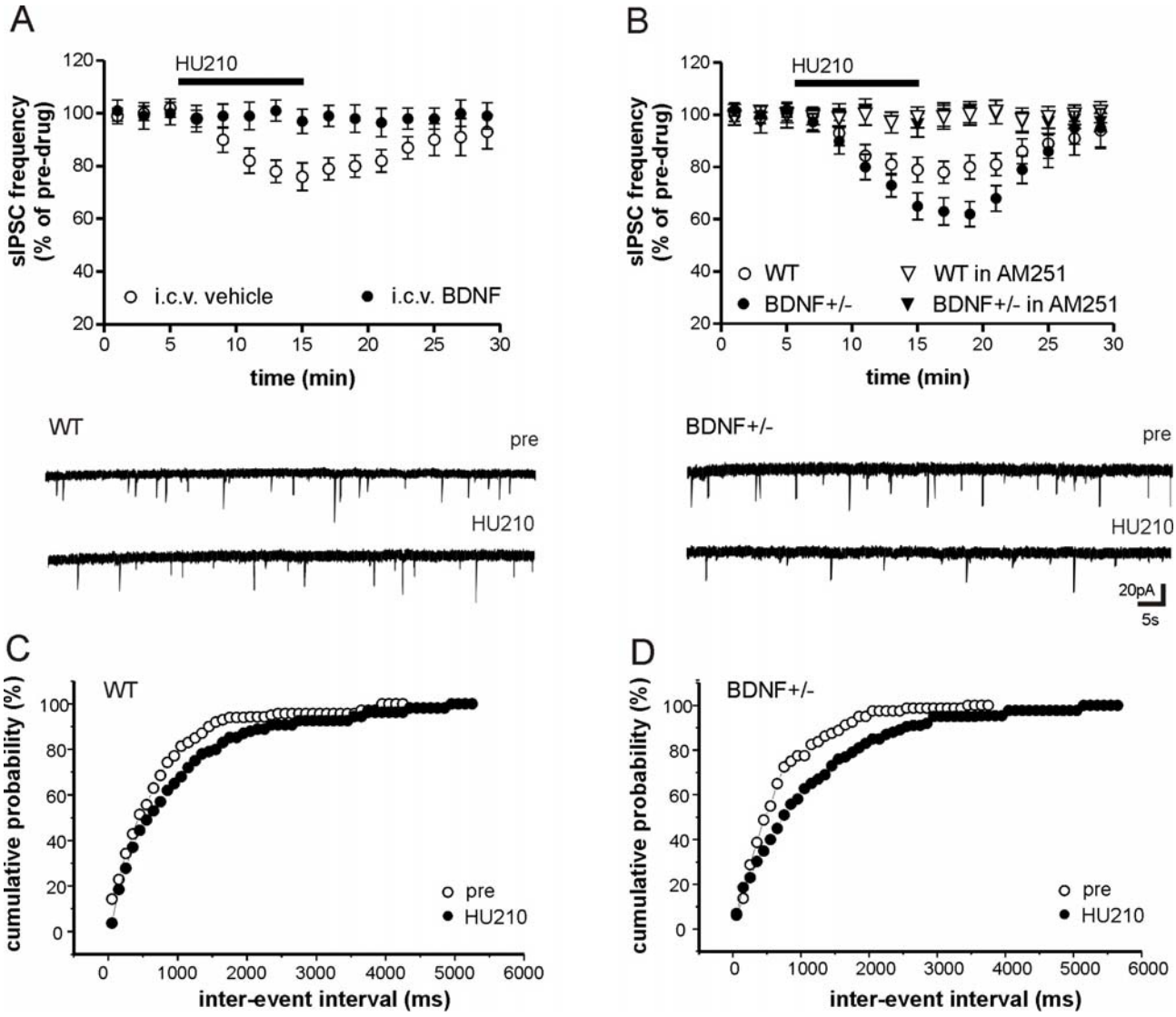

Figure 12. In vivo manipulations of BDNF levels alters the sensitivity of CB1 receptors on GABA synapses. A. HU210 was ineffective in reducing mIPSC frequency in mice receiving BDNF *via* a single i.c.v injection. B. HU210-mediated inhibition of sIPSC frequency was selectively potentiated in striatal neurons from mice with partial genetic BDNF depletion (BDNF+/- mice). This effect was completely prevented by preincubation with the CB1 receptors antagonist AM251. The electrophysiological traces below are examples of voltage clamp recordings before and during the application of HU210 in control and BDNF+/- mice. C, D. Cumulative distribution of sIPSC inter-event interval recorded from WT (C) and BDNF+/- (D) mice before and during the application of HU210.

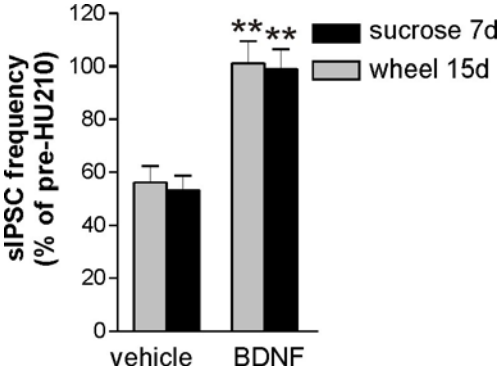

Figure 13. BDNF is able to fully block sensitized CB1 receptors on GABA synapses. The graph shows that HU210-induced reduction of sIPSC were potentiated after 7 days of sucrose exposure and after 15 days of exposure to running wheel. I.c.v. injection of BDNF, at the end of sucrose and wheel exposure protocols, fully blocked the HU210-mediated inhibition of sIPSC.

DISCUSSION

The present study demonstrates that natural reward and stressful events cause significant adaptations of cannabinoid CB1 receptor-mediated control of GABA transmission in the striatum.

The synaptic responses to the selective cannabinoid CB1 receptor agonist HU210 were normal after a single day of exposure to running wheel or to sucrose, but remarkably potentiated after 7 or 15 days of treatments, whereas they were reduced after a single stressful episode, and fully abolished after 3 and 7 days of stress exposure, indicating that cannabinoid receptors progressively adapt during these environmental manipulations.

Furthermore, the synaptic adaptations caused by both natural reward and stress exposure were slowly reversible after the discontinuation of the treatments, indicating that they were not irreversible.

Stress exposure, running wheel or sucrose selectively altered the sensitivity of cannabinoid receptors controlling GABA transmission in the striatum, while the sensitivity of glutamate synapses to CB1 receptor stimulation was unaltered, indicating the existence of differential regulation mechanisms of distinct cannabinoid receptors. These findings are compatible with the preferential location of CB1 receptors on GABAergic nerve terminals described in the striatum (Narushima *et al*, 2007; Uchigashima *et al*, 2007). In line with this observation, we have recently reported that the sensitivity of GABA synapses to the cannabinoid receptor stimulation was selectively reduced in Huntington's disease mice (Centonze *et al*., 2005), and selectively enhanced in rats chronically treated with cocaine (Centonze *et al*., 2007a). Our data also show that the tested environmental manipulations do not result in a widespread dysregulation of the presynaptic control of striatal GABA synapses, since they did not alter the sensitivity of sIPSCs to the stimulation of other G-alpha-i/o-coupled receptors, such as GABAB receptors.

We have also shown that social stress, running wheel or sucrose altered the synaptic effects not only of the exogenous cannabinoid HU210 but also of endocannabinoids mobilized in the striatum in

response to mGlu 5 receptor stimulation. This finding suggests that these alterations of cannabinoid transmission may have relevant synaptic consequences during the physiological activity of the striatum, which is mainly driven by glutamate inputs originating from the cortex and the thalamus (Wilson and Kawaguchi, 1996; Stern *et al*, 1998).

Since BDNF also play a role in the emotional consequences of stress and of rewarding experiences, we have exstended our study to address the functional interplay between BDNF and cannabinoid CB1 receptors in the striatum. The results of the present study show that BDNF is a strong regulator of CB1receptor function in the striatum, providing evidence for a novel action of this neurotrophin in this brain area. Again the inhibition of CB1 receptor activity by BDNF was restricted to GABA synapses. Our data also show that BDNF does not result in a widespread dysregulation of the presynaptic control of striatal GABA synapses, since it did not alter the sensitivity of sIPSCs to the stimulation of other G-alpha-i/o-coupled receptors, such as GABAB receptors. Finally, pharmacological inhibition of tyrosine kinase activity prevented the action of BDNF on CB1 receptor on GABA synapses, confirming the crucial role of TrkB in the synaptic effects of this neurotrophin.

The relationship between stress and endocannabinoid system is complex, since it has been reported that stressful events increase endocannabinoid levels in several brain areas. Activation of cannabinoid receptors mediates specific 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). Stimulation of cannabinoid receptors is also critical for the activation of the amygdala during stress (Patel *et al*., 2005a). On the other hand, it has been shown that stress impairs cannabinoid CB1 receptor-mediated transmission (Hill *et al.*, 2005), and evidence exists that this effect may mediate some aspects of the stress response. It has also been shown, in fact, that stimulation of CB1 receptors, reduces, rather than enhances, the expression of active escape behavior during an acute stress

episode (Patel *et al*., 2005b), attenuates stress-induced anhedonia (Rademacher and Hillard, 2007) and other stress-induced depressive manifestations (Gobbi *et al*., 2005), and reduces hippocampus-dependent cognitive impairment induced by chronic stress (Hill *et al*., 2005). Furthermore, genetic and pharmacological inactivation of CB1 receptors promotes passive stress-coping behavior (Steiner et al., 2008) and stress-induced motor inhibition (Fride et al., 2005), while pharmacological stimulation of the endocannabinoid system reduces the suppression of hippocampal cell proliferation and the increase in defensive behaviors seen in rats exposed to predator odor (Hill *et al*., 2006). To reconcile these discrepant results, it can be proposed that duration, type and context of the stress paradigm are all important to determine whether cannabinoids have a prevailing facilitatory or inhibitory role in the resulting stress-induced behavioral changes, possibly because of a differential activation of the hypothalamus-pituitary-adrenal axis during different stress conditions.

Some evidence supports the concept of a bidirectional functional relationship between glucocorticoids and the endocannabinoid system. Hypothalamus-pituitary-adrenal axis, in fact, regulates endocannabinoid production (Di *et al*., 2005; Malcher-Lopes *et al*., 2006) and CB1 receptor expression (Hill *et al*., 2008) in the brain and it is, in turn, regulated by endocannabinoids. Corticosteroids released in response to the activation of the hypothalamic-pituitary-adrenal axis play a major role in the synaptic defects of stressed animals, since these alterations were mimicked by corticosterone injections and were fully prevented by pharmacological blockade of glucocorticoid receptors. In this respect, a recent study showed that CB1 receptors are under a negative regulation by glucocorticoids in the hippocampus, and suggest that hippocampal cannabinoid CB1 receptor signaling could be reduced under conditions associated with hypersecretion of glucocorticoids, such as chronic stress (Hill *et al*., 2008).

Furthermore, inactivation of cannabinoid CB1 receptors increases adrenocorticotropin and corticosterone plasma concentrations (Manzanares *et al*., 1999; Haller *et al*., 2004), while stimulation of these receptors has an opposite effect (Patel *et al*., 2004). In this respect, a novel

finding of our study consists in the demonstration that stress-induced loss of cannabinoid CB1 receptor sensitivity in the striatum can be fully prevented by inhibiting glucocorticoid receptors and mimicked by enhancing glucocorticoid levels.

The effects on cannabinoid responses observed in this study disappeared within a few days after the cessation of the stress sessions. These data are consistent with previous electrophysiological work showing that stress-induced defects of hippocampal synaptic plasticity are labile and undergo a passive run-down when the animals are not longer exposed to stress (Yang *et al*., 2004).

Of note, the recovery of stress-induced synaptic defects were accelerated when the mice were given access to a running wheel or to sucrose consumption, which function as potent natural rewarding stimuli (Werme *et al*., 2000, 2002; Mahler *et al*., 2007; Rademacher and Hillard, 2007). Enriched environment and novelty exploration has been reported to accelerate the reversal of stress-induced synaptic defects in the hippocampus (Yang *et al*., 2006; Yang *et al*., 2007), a result consistent with our findings.

Our previous report indicated that hypersensitivity of striatal GABA synapses to HU210 tightly correlated with the rewarding properties of cocaine, since this synaptic alteration appeared only when a conditioned place preference (CPP) was induced. A single cocaine exposure which failed to induced CPP, conversely, also failed to sensitize striatal cannabinoid receptors (Centonze *et al*, 2007a). Notably, the CPP procedure selectively examines the positive reinforcing properties of addictive compounds (Acquas and Di Chiara, 1994; Tzschentke, 1998; Le Foll and Goldberg, 2005), and previous behavioral studies indicated that the (endo)cannabinoid system, rather than contributing to the hedonic or psychomotor effects of cocaine consumption, is involved in the generation and maintenance of reward-based addictive behavior (De Vries and Schoffelmeer, 2005; Le Foll and Goldberg, 2005; Parolaro *et al*, 2005). Here, we have observed that voluntary wheel running and sucrose consumption share with chronic cocaine the common neurobiological background of increasing the sensitivity of cannabinoid receptors modulating GABA transmission

in the striatum (Centonze *et al*, 2007a). It is conceivable, therefore, that this alteration might represent a synaptic correlate of reward-based behavior. Evidence of the rewarding and potentially addictive properties of running wheel is currently accumulating. Rodents, in fact, are highly motivated to gain access to running wheels and display CPP to an environment associated with wheel running (Iversen, 1993; Belke, 1997; Lett *et al*, 2000; de Visser *et al*, 2007). Furthermore, animals that run long distances daily exhibit withdrawal signs when access to the running wheels is denied (Hoffmann *et al*, 1987), and display increased vulnerability for cocaine self-administration and reinstatement after abstinence (Larson and Carroll, 2005). On the other hand, intense rewarding properties of sucrose have also been reported (Lenoir *et al*, 2007), and similar activation of the central reward pathway has been found after sweet tasting (Mark *et al*, 1991; Hajnal *et al*, 2004) and drugs of abuse (Di Chiara and Imperato, 1988; Pontieri *et al*, 1996). Furthermore, some behavioral and neurochemical signs of opiate withdrawal can be precipitated by naloxone in rats with sugar overconsumption (Colantuoni *et al*, 2004).

Wheel running, however, also has a strong motor activating effect in mice, and the relationship between this experimental procedure and the mechanisms of reward is unclear, especially when animals are housed in groups, as in the case of the present study. Similarly, at least part the effects of sucrose on cannabinoid CB1 receptors might also be unrelated to its rewarding properties, and reflect for example increased caloric assumption. However, we found striking similarities between the neurophysiological and behavioral effects of running wheel and of sucrose, which has reinforcing properties but not motor activating effects. These findings suggest therefore that the alteration of cannabinoid CB1 receptor function here described likely reflect the emotional effects of the two experimental paradigms, as also indicated by the evidence that cannabinoid CB1 receptor blockade abolishes the protective action of both running wheel and of sucrose on stress-induced behavior. The commonalities among running wheel, sucrose consumption, and cocaine exposure might suggest that natural and drug-induced rewards trigger a common synaptic adaptation involving cannabinoid CB1-mediated transmission in the striatum. The importance of this

adaptation in the behavioral consequences of the activation of the central reward pathway is evident when considering that exercise activates the endocannabinoid system also in humans (Sparling *et al*, 2003), and that the motivation for sweet food (Simiand *et al*, 1998; Cota *et al*, 2003; Ward and Dykstra, 2005; Mahler *et al*, 2007) and the rewarding properties of cocaine (Chaperon *et al*, 1998) are both significantly attenuated after blockade of cannabinoid CB1 receptors.

The regulation of striatal CB1 receptor function might be important for the effect of BDNF on mood control. The BDNF synaptic effects here described mirror the alteration of the sensivity of striatal synapses to the activation of the CB1 receptors described so far, and are also in good agreement with the evidence that intrastriatal infusion of BDNF induces a depressive effect (Eisch *et al.*, 2003), while inhibition of BDNF–TrkB signaling in this brain area elicits anti-depressive actions (Eisch *et al*., 2003), and contrasts the behavioral consequences of the same stress protocol causing striatal CB1 receptors downregulation on GABA synapses in mice performed in this work (Berton *et al.*, 2006).

The important role of extra-striatal BDNF in mood control, and especially the evidence that hippocampal BDNF infusion causes antidepressant-like effects (Siuciak *et al*., 1997; Shirayama *et al*., 2002), prevented us from analyzing the emotional consequences of i.c.v. BDNF administration, to address the behavioral counterpart of our synaptic findings. Further lines of evidence, however, support the idea that BDNF-mediated inhibition of CB1 receptor function represent a synaptic correlate of the anxious depressive behavior induced by the activation of BDNF-TrkB signaling in the striatum (Eisch *et al*., 2003; Berton *et al*., 2006).

Enhancement of cAMP signaling in the striatum has been associated with increased anxious-depressive behavior in mice (Favilla *et al*., 2008; Kim *et al*., 2008; Zhang *et al*., 2008), and confirms the relevance of our findings for the pathophysiology of emotional disorders. CB1 receptors, in fact, reduce transmitter release by inhibiting cAMP levels in presynaptic nerve terminals (Howlett *et al*., 2004), implying that BDNF-mediated loss of CB1 receptors sensitivity

results in enhanced cAMP signaling in striatal nerve terminals. GABA-mediated inhibition of striatal neuron activity possibly disrupts a circuitry normally limiting fearful or anxiety-related behaviors (Rogan *et al*., 2005). Furthermore, the effects of *in vivo* manipulations contrasting anxiety in stressed animals, are associated with increased sensitivity of striatal CB1 receptors, and are fully blocked by treatment with BDNF (present study).

CONCLUSIONS

In conclusion, here we have identified an association between stress-induced emotional alterations and the downregulation of striatal cannabinoid CB1 receptor-mediated transmission, and found that this alteration was sensitive to the inhibition of glucocorticoid receptors and to the activation of the central reward system.

Our present results also show that voluntary exercise and sucrose consumption exert protective effects against the motor behavior induced by stress, and that the sensitization of cannabinoid CB1-mediated synaptic responses in the striatum are likely involved in these effects. Targeting cannabinoid CB1 receptors or endocannabinoid metabolism might be a valuable option to treat stress-associated neuropsychiatric conditions and anxiety disorders.

Finally the results of the present study show that BDNF is a strong regulator of CB1 receptor function in the striatum, providing evidence for a novel action of this neurotrophin in this brain area. with potentially relevant implications for important cognitive and behavioral functions.

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APPENDIX