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BDNF impairment in the hippocampus is related to enhanced despair behavior in CB_1 knockout mice

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Abstract

Stress can cause damage and atrophy of neurons in the hippocampus by deregulating the expression of neurotrophic factors that promote neuronal plasticity. The endocannabinoid system represents a physiological substrate involved in neuroprotection at both cellular and emotional levels. The lack of CB_1 receptor alters neuronal plasticity and originates an anxiety-like phenotype in mice. In the present study, CB_1 knockout mice exhibited an augmented response to stress revealed by the increased despair behavior and corticosterone levels showed in the tail suspension test and decreased brain derived neurotrophic factor (BDNF) levels in the hippocampus. Interestingly, local administration of BDNF in the hippocampus reversed the increased despair behavior of CB_1 knockout mice, confirming the crucial role played by BDNF on the emotional impairment of these mutants. The neurotrophic deficiency seems to be specific for BDNF as no differences were found in the levels of nerve growth factor and NT-3, two additional neurotrophic factors. Moreover, BDNF impairment is not related to the activity of its specific tyrosine kinase receptor or the activity of the transcription factor cAMP responsive element binding. These results suggest that the lack of CB_1 receptor originates an enhanced response to stress and deficiency in neuronal plasticity by decreasing BDNF levels in the hippocampus that lead to impairment in the responses to emotional disturbances.

Keywords: brain derived neurotrophic factor, CB_1 cannabinoid receptor, corticosterone, cyclic AMP responsive element binding, stress, tyrosine kinase receptor.

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Stressful events induce neurotrophins activation in the brain that is hypothesized to provide protection against neuronal damage and to stimulate sprouting and synaptic reorganization, enabling neural networks to cope with these stimuli (Lindvall *et al.* 1994). However, prolonged and severe stress exposure can lead to neuronal atrophy and cell loss by inducing neurotrophic impairment in key limbic regions controlling emotional responses (Duman and Monteggia 2006). Limbic system regulates the hypothalamic-pituitary–adrenal (HPA) axis activity through inhibitory inputs from the hippocampus and the frontal cortex and stimulatory action from the amygdala, participating in the appropriate response to stress (Herman *et al.* 2005). Among these brain structures, the hippocampus is particularly sensitive to stress-induced alterations because of the high density of glucocorticoid receptors in this specific brain region (McEwen 1999). In this sense, sustained elevations of glucocorticoids can induce damage in hippocampal neurons by reducing dendrite

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Abbreviations used: BDNF, brain derived neurotrophic factor; CREB, cAMP responsive element binding; HPA, hypothalamic-pituitary–adrenal; NGF, nerve growth factor; rhBDNF, recombinant human BDNF; TBS, Tris-buffered saline; TrkB, tyrosine kinase receptor; TST, tail suspension test.

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branching and the number of dendritic spines (Sapolsky 2000a). Therefore, these changes in synaptic connectivity of hippocampal neurons induced by stress might reduce the inhibitory control that this structure exerts on the HPA axis, leading to a positive feedback process with pathological consequences, such as hippocampal volume reduction and cognitive impairments (Radley and Morrison 2005; Bremner 2006).

Brain derived neurotrophic factor (BDNF) is one of the most prevalent factors that modulates plasticity and survival of adult neurons (Huang and Reichardt 2001). Several pre-clinical and clinical evidences demonstrate that BDNF plays a role in the pathophysiology of stress-related mood disorders (Duman and Monteggia 2006). The expression of BDNF is partially regulated by the transcription factor cAMP responsive element-binding (CREB) (Conti *et al.* 2002), which represents a central integrator of signaling from a number of extracellular stimuli that influence neuronal plasticity and survival (Duman 2002). Most neuronal effects of BDNF are mediated through the high-affinity tyrosine kinase receptor (TrkB). BDNF binding to TrkB activates several signaling pathways (Kaplan and Miller 2000), which subsequently induce biological responses including protection against stress-induced neuronal damage.

The endocannabinoid system represents an important substrate for the control of emotional behavior (Valverde 2005). The CB_1 receptor, the most abundant cannabinoid receptor in the brain, is expressed in all of the main brain structures involved in stress-related behaviors such as the hypothalamus, amygdala, limbic system, habenula, cortex, and hippocampus (Matsuda *et al.* 1993) and participates in the control of the HPA axis (Barna *et al.* 2004; Patel *et al.* 2004; Cota *et al.* 2007). Moreover, the lack of CB_1 receptor induced neuronal plasticity impairments as demonstrate the loss of neurons (Bilkei-Gorzo *et al.* 2005) and defective neurogenesis in the hippocampus (Jin *et al.* 2004) and the increased susceptibility to neurotoxic insults described in CB_1 knockout mice (Marsicano *et al.* 2003).

The aim of the present study was to investigate the role of the hippocampal BDNF in the emotional responses of mice lacking CB_1 receptor considering the participation of the endocannabinoid system in the stress-related responses and the relevance of the neurotrophic-induced plasticity in the hippocampal response to stressful events.

Materials and methods

Animals

All the experiments were carried out in male CB_1 knockout mice and wild-type littermates 8–12 weeks old at the beginning of the experiments. The generation of mice lacking CB_1 cannabinoid receptor was described previously (Ledent *et al.* 1999). To homogenize the genetic background of mice, the first generation heterozygous was bred for 30 generations on a CD1 background, with selection for the mutant CB_1 gene at each generation. Beginning with the 30th generation backcrossing mice, heterozygote–heterozygote mating of CB_1 knockout mice produced wild-type and knockout littermates for subsequent experiments. All animals used in a given experiment were matched for age and weight. Mice were housed five per cage in a temperature (21 ± 1°C) and humidity-controlled (55 ± 10%) room with a 12:12 hours light/dark cycle (light between 08:00 and 20:00 hours) with food and water *ad libitum*. The observer was blind to genotype and/or treatment in all the experiments. Animal procedures were conducted according to standard ethical guidelines (European Communities Council Directive 86/609/EEC) and approved by the Local Ethical Committee (IMAS-IMIM/UPF).

Drugs

Recombinant human brain derived neurotrophic factor (rhBDNF) was purchased from Promega Corporation® (Madison, WI, USA). rhBDNF was dissolved in a filtered Ringer solution (148 mmol/L NaCl, 2.7 mmol/L KCl, 1.2 mmol/L $CaCl_2$, 0.8 mmol/L $MgCl_2$ in distilled water, all the components provided by Scharlau Chemie, Paris, France). The cell labeling CellTracker™ CM-DiI was supplied by Molecular Probes™ (Eugene, OR, USA).

Experimental procedure

Stress exposure

Animals were exposed to the tail suspension test (TST). Mice were individually suspended by adhesive tape 1 cm from the tip of the tail 50 cm above a bench top for a 6 min period as described by Steru *et al.* (1985). The time that the animal was totally inactive during this period was recorded. After the TST exposure, animals were individually housed and 1 h later were killed. Blood samples were collected, and brain areas were dissected and immediately frozen at −80°C.

Basal measurements of serum corticosterone, BDNF, and CREB levels were obtained from CB_1 knockout mice and wild-type littermates which were killed immediately after taking them from their home cage.

BDNF hippocampal microinjection

Recombinant human BDNF was locally administered into the hippocampus (0.25 µg/side). Mice were first anesthetized with a ketamine/xylazine mixture 5 : 1; 0.10 mL/10 g body weight, i.p.) and subsequently mounted in a stereotaxic frame (KOPF Instruments, Tujunga, CA, USA). The coordinates (AP: −1.9; ML: ±1.8, DV: −2.3, expressed in mm) for the cannuli (7 mm long, 30 gauge) implantation into the hippocampus were taken from bregma and the skull surface according to the stereotaxic atlas (Paxinos and Franklin 1997). Mice were administered bilaterally into the hippocampus. Each animal received 1 µL/side of vehicle or rhBDNF and 0.2 µL/side of the cell labeling CM-DiI. The infusion was carried out during 5 min and the cannula was removed 2 min after the end of the infusion. To evaluate the effect of hippocampal rhBDNF microinjection, TST was performed, as described previously, 3 days after the surgery. After TST, mice were killed and the brains were

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removed and frozen. Coronal sections (40 μm) of the brain were cut on a cryostat at −26°C and a red fluorescent light was used to reveal the exact location of CM-DiI at the site of infusion, although small amounts of CM-DiI signal were observed in practically all the anterior–posterior length of the hippocampus. Data from mice with wrong location of the cannuli were removed from the study.

Preparation of samples

Blood samples

Blood samples were allowed to coagulate and were then centrifuged (800 *g*, 10 min at 4°C). Serum was recovered and frozen at −80°C until corticosterone measurements.

Brain areas processing

Frozen brain areas were dounce-homogenized in lysis buffer (137 mmol/L NaCl; 20 mmol/L Tris–HCl, pH 8.0; 1% NP-40; 10% glycerol; 1 mmol/L sodium vanadate; 5 mmol/L sodium pyrophosphate; 100 mmol/L NaF; 40 mmol/L glycerol phosphate; 1 mmol/L phenylmethylsulfonyl fluoride; 0.15 μmol/L aprotinine; 11 μmol/L leupeptine; and 1.5 μmol/L pepstatine) to prepare protein extract. Hippocampus and frontal cortex were homogenized in 30 μL lysis buffer/mg wet weight whereas amygdala was homogenized in 50 μL lysis buffer/mg wet weight. After 20 min of incubation in agitation at 4°C, samples were centrifuged during 30 min at 16 000 *g*, and the supernatant was recovered and stored at −80°C. Protein content was determined by using the DC Protein Assay (Bio-Rad, Barcelona, Spain) following manufacturer's instructions.

Free-floating brain sections

Mice were deeply anesthetised by i.p. injection (0.2 mL/10 g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) prior to intracardiac perfusion of 4% paraformaldehyde in 0.1 mol/L Na_2HPO_4/NaH_2PO_4 buffer, pH 7.5, delivered with a peristaltic pump at 19 mL/min for 5 min. Brains were removed and post-fixed overnight at 4°C in the same fixative solution. Brain free-floating sections (30 μm) through the hippocampus were obtained with a vibratome (Leica, France) and kept in a cryoprotection solution containing 30% ethylene glycol, 30% glycerol, and 0.1 mol/L Na_2HPO_4/NaH_2PO_4 buffer at −20°C until they were processed for immunohistochemistry.

Corticosterone levels measurement

Serum corticosterone levels were measured by using the Coat-A-Count® Rat Corticosterone (Diagnostic Product Corporation, Los Angeles, CA, USA) which is a solid-phase ^{125}I radioimmunoassay designed for the quantitative measurement of corticosterone in rodent serum with a lower sensitivity limit of detection of 5.7 ng/mL. Data were expressed as mean ± SEM.

Neurotrophic quantification

The BDNF Emax™ Immunoassay System (Promega Corporation®) was used to quantify the levels of BDNF protein. Prior to each assay, samples were diluted and acid treated to adjust the amount of BDNF to the standard curve and to increase the detectable amount of free BDNF in solution by dissociating it from its proforms or receptors (Okragly and Haak-Frendscho 1997). Samples from hippocampus and frontal cortex were diluted 1 : 80 (vol/vol) and amygdala in 1 : 50 (vol/vol) in lysis buffer before the assay. All the samples were acidified with 1 μL 1 mol/L HCl/50 μL sample and after 15 min of incubation at 21 ± 1 °C, samples were neutralized with the same amount of NaOH 1 mol/L. MaxiSorp™ 96 well plates (Nunc™, Roskilde, Denmark) were used for antibody coating and ELISA was carried out according to manufacturer's instructions. BDNF levels were normalized to the total amount of protein from each individual sample. A similar procedure was conducted to measure nerve growth factor (NGF) and NT-3 levels by using specific immunoassays (Promega Corporation®). Data were calculated as percentage of respective control and were expressed as mean ± SEM.

BDNF immunohistochemistry

Free-floating sections were washed in 0.25 mol/L Tris-buffered saline (TBS), incubated in TBS containing 3% H_2O_2 and 10% methanol for 10 min, and thoroughly washed again in TBS. Tissue permeabilization was facilitated by the incubation with 0.2% TX-100 for 15 min at 21 ± 1 °C. Sections were incubated with the rabbit anti-BDNF antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany, 1 : 50) overnight at 4°C and subsequently washed in TBS. Then, sections were incubated in biotinylated goat anti-rabbit IgG (1 : 400) for 2 h. After being washed, the sections were incubated for 90 min in avidin–biotin-peroxidase complex solution (final dilution, 1 : 50; Vector Laboratories, Peterborough, UK). Sections were then washed in TBS and in TB (0.25 mol/L Tris, pH 7.5), placed in a solution of TB containing 0.1% 3,3′-diaminobenzidine (30 mg/100 mL), and developed by H_2O_2 addition (0.02%). After processing, the tissue sections were mounted onto gelatin-coated slides and dehydrated through alcohol to xylene for light microscopic examination.

CREB and TrkB protein levels quantification

Cyclic AMP responsive element binding, phospho-CREB and TrkB levels were quantified by western blotting. Equal amounts of lysates (40–50 μg per lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12.5%) before electrophoretic transfer onto polyvinylidene difluoride membrane (CREB detection) or nitrocellulose membrane (phospho-CREB and TrkB detection) (Bio-Rad). Membranes were blocked for 1 h at 21 ± 1 °C in TBS (100 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) with 0.1% Tween 20, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF (0.5 mol/L) and 5% non-fat milk (CREB detection) or 3% non-fat milk (phospho-CREB detection). Afterwards, membranes were incubated overnight with the primary antibodies (CREB, 1 : 800, Upstate, Billerica, MA, USA; phospho-serine-133-CREB, 1 : 800, Upstate; TrkB (794) sc-12, 1 : 50, Santa Cruz Biotechnology Inc.; GAPDH, 1 : 2000, Santa Cruz Biotechnology Inc.). Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit (Sigma, Tres Cantos, Madrid, Spain; diluted 1 : 60 000, 1 h at 21 ± 1 °C) and visualized by enhanced chemiluminescence detection (ECL Advance™; Amersham Biosciences, Piscataway, NJ, USA). After autoradiography, the films were scanned and the integrated optical density values corresponding to the relevant immunoreactive bands were obtained by image analysis (Image Gauge 3.12; Fuji Photo Film Co., Ltd, Barcelona, Spain). The value of active phospho-CREB was normalized to the amount of total CREB in the same sample and expressed as a percentage of control treatment. To assess changes in

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CREB and TrkB levels, total CREB and TrkB content was normalized to the GAPDH content in the same sample and shown as a percentage of control treatment. Data were expressed as mean ± SEM.

Statistical analyses

Data were analyzed by two-way ANOVA with genotype and stress exposure or treatment as between factors, followed by one-way ANOVA when required. Comparisons between genotypes in the TST, NGF, and NT-3 levels in the hippocampus and BDNF levels in frontal cortex and amygdala were analyzed by one-way ANOVA. Comparisons were considered statistically significant when the level of significance was < 0.05.

Results

Enhanced response to stress in CB_1 knockout mice

The exposure to TST induced a higher immobility time in CB_1 knockout mice than in wild-type littermates ($F_{(1,13)} = 9.412$, $p < 0.01$), revealing an increased despair behavior in mice lacking CB_1 receptor (Fig. 1a).

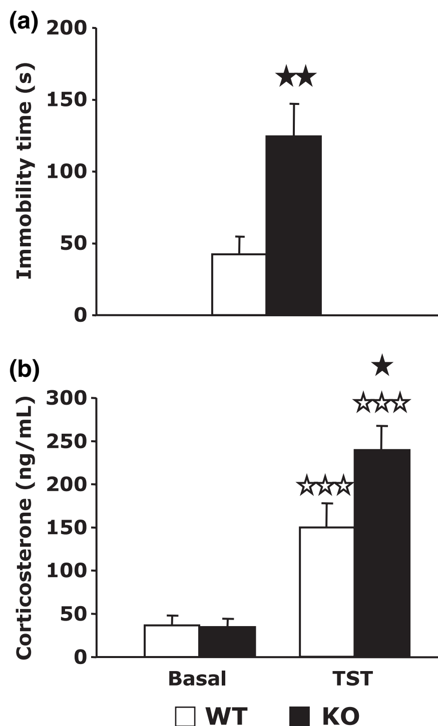

Fig. 1 Enhanced response to stress in CB_1 knockout mice. (a) Increased despair behavior of CB_1 knockout (filled bars) compared with wild-type (open bars) mice on the tail suspension test (TST). (b) Serum corticosterone levels were increased in CB_1 knockout mice 1 h after exposure to the TST compared with wild-type mice but not in basal conditions. Data are expressed as mean ± SEM (n = 8–10). ☆☆☆$p < 0.001$, when compared with basal group of the same genotype. ★$p < 0.05$, ★★$p < 0.01$, comparison between genotypes (one-way ANOVA).

The corticosterone release produced by the exposure to TST was higher in mutant mice than in wild-type animals, although both genotypes exhibited similar levels of serum corticosterone under basal conditions, evidencing an enhanced neuroendocrine response to stress in mutant mice (Fig. 1b). Two-way ANOVA revealed a significant effect of stress exposure ($F_{(1,28)} = 65.819$, $p < 0.001$), genotype ($F_{(1,28)} = 5.012$, $p < 0.05$) and interaction between these two factors ($F_{(1,28)} = 5.336$, $p < 0.05$). Subsequent one-way ANOVA calculated for the response to stress indicated an effect of stress exposure in wild-type ($F_{(1,14)} = 16.640$, $p < 0.001$) and CB_1 knockout mice ($F_{(1,14)} = 54.970$, $p < 0.001$). One-way ANOVA calculated for genotype effect showed higher levels of corticosterone in CB_1 knockout mice exposed to stress compared to wild-type animals ($F_{(1,12)} = 4.754$, $p < 0.05$) but not in basal conditions.

BDNF decreased levels in hippocampus of CB_1 knockout mice

BDNF protein quantification (ELISA)

CB_1 knockout mice showed decreased BDNF levels in the hippocampus at basal conditions (wild-type: 651 ± 51 ng BDNF/g wet weight; CB_1 knockout: 451 ± 37 ng BDNF/g wet weight) and after stress exposure (Fig. 2a), indicating an impairment in the neurotrophic support in mutant mice. Two-way ANOVA revealed a significant effect of stress exposure ($F_{(1,31)} = 72.509$, $p < 0.001$) and genotype ($F_{(1,31)} = 16.511$, $p < 0.001$), but not interaction between these two factors. Subsequent one-way ANOVA indicated an increase of BDNF levels in response to stress in both wild-type ($F_{(1,16)} = 28.060$, $p < 0.001$) and CB_1 knockout mice ($F_{(1,15)} = 84.631$, $p < 0.001$). Comparisons between genotypes revealed lower BDNF levels in CB_1 knockout mice under basal conditions ($F_{(1,17)} = 6.855$, $p < 0.05$) and after stress exposure ($F_{(1,14)} = 9.098$, $p < 0.001$).

The analysis of BDNF content in frontal cortex and amygdala revealed no differences between genotypes (Fig. 2c), suggesting specific brain area BDNF impairment in CB_1 knockout mice.

The neurotrophic deficiency in the hippocampus of CB_1 knockout mice seems to be specific for BDNF as the analysis of other neurotrophic factors such as NGF (wild-type 100.0 ± 8.3; knockout 107.8 ± 10.8) and NT-3 (wild-type 100.0 ± 7.3; knockout 99.9 ± 12.5) indicated no differences between genotypes (Fig. 2d).

Immunohistochemistry

CB_1 knockout mice hippocampal BDNF impairment revealed by ELISA quantification was confirmed by the microscopic examination of the BDNF immunoreactivity in brain sections (Fig. 2b). The BDNF deficiency was homogeneously observed in the all the areas of the hippocampus.

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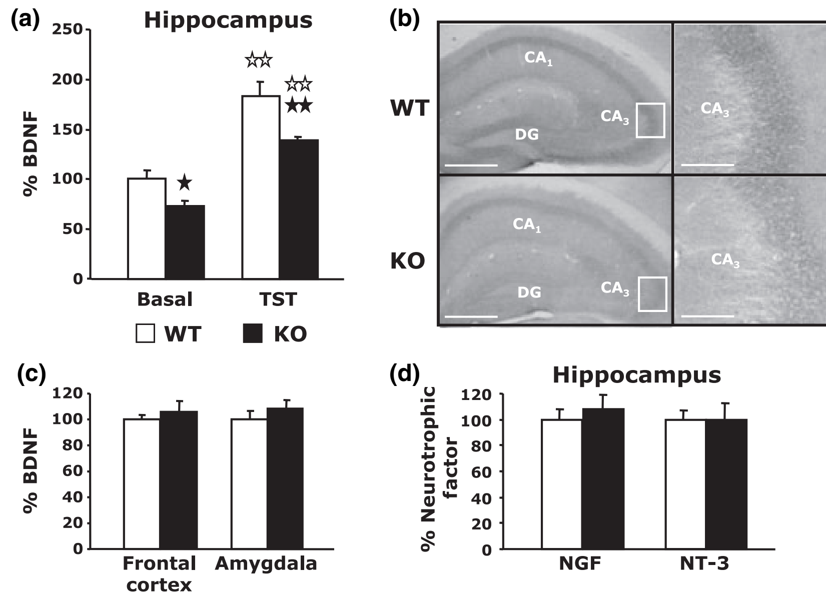

Fig. 2 Brain derived neurotrophic factor (BDNF) impairment in the hippocampus of CB_1 knockout mice. (a) Decreased BDNF levels measured by ELISA in the hippocampus of CB_1 knockout (filled bars) compared with wild-type (open bars) mice in basal conditions and after stress exposure. (b) Representative images of the BDNF immunoreactivity in coronal sections of the hippocampus of wild-type (upper panel) and CB_1 knockout (lower panel) mice in basal conditions. Right panels illustrate higher-magnification pictures of the CA_3 region of the hippocampus. Scale bar represents 100 μm in the left panels and 50 μm in right panels. DG, dentate gyrus. (c) Measurement of the BDNF levels by ELISA in the frontal cortex (pre-limbic and motor cortex) and amygdala revealed no difference between genotypes in basal conditions. (d) Neurotrophic deficiency in the hippocampus of CB_1 knockout mice seems to be specific for BDNF as there is not difference between genotypes in the nerve growth factor (NGF) and NT-3 protein contents measured by ELISA. Data are expressed as mean ± SEM of the percentage of neurotrophic factors respect the wild-type control group (n = 8–10). ☆☆$p < 0.01$, when compared with control group of the same genotype (one-way ANOVA). ★$p < 0.05$, ★★$p < 0.01$, comparison between genotypes (one-way ANOVA).

CREB and TrkB quantification

The analysis of hippocampal samples revealed no differences between genotypes in ratio phospho-CREB/CREB (Fig. 3a and b) or in the amount of the TrkB receptor (Fig. 3a and c). No significant differences were either observed in the total amount of CREB in hippocampal samples (data not shown).

Reversal of CB_1 knockout increased despair behavior by local BDNF hippocampal microinjection

Local administration of BDNF (0.25 μg/side) into the hippocampus reversed the immobility induced by the TST in both wild-type and CB_1 knockout mice (Fig. 4). Two-way ANOVA indicated a significant treatment effect ($F_{(1,35)}$ = 17.060, $p < 0.001$), without genotype effect, nor interaction between these two factors. One-way ANOVA revealed significant reduction in the immobility time of BDNF-treated wild-type ($F_{(1,17)}$ = 5.511, $p < 0.05$) and CB_1 knockout mice ($F_{(1,18)}$ = 11.877, $p < 0.01$). Comparisons between genotypes showed a significant increase in the immobility time in vehicle-treated CB_1 knockout mice ($F_{(1,18)}$ = 5.075, $p < 0.05$), but no differences were observed between both groups of BDNF-treated mice.

Discussion

The present study reveals BDNF impairment in the hippocampus of CB_1 knockout mice. This neurotrophic factor plays a key role in the enhanced response to stress observed in mutant mice as BDNF administration in the hippocampus completely reversed the increased despair behavior exhibited by CB_1 knockout mice in the TST.

Mice lacking CB_1 receptor exhibited higher immobility time than wild-type littermates when exposed to the TST. This behavioral paradigm is considered to evidence a state of despair related to mood disorders (Nestler *et al.* 2002). The participation of CB_1 receptor in the neurobiological mechanisms involved in mood disorders and stress is supported by the present data and previous findings reporting an increased sensitivity of CB_1 mutant mice to exhibit anhedonia after chronic unpredictable mild stress exposure (Martín *et al.* 2002), increased passive stress-coping behaviors in the forced swimming test (Steiner *et al.* 2007) and a higher vulnerability to behavioral inhibition after repeated or acute severe stress (Fride *et al.* 2005), among others (Hill and Gorzalka 2005). The enhanced despair behavior revealed in CB_1 knockout mice when exposed to a single TST was

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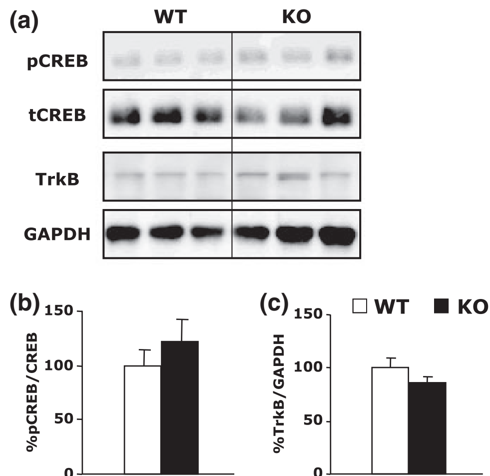

Fig. 3 Brain derived neurotrophic factor (BDNF) basal impairment is independent of the transcription factor cAMP responsive element binding (CREB) activity or the density of the specific tyrosine kinase receptor (TrkB). (a) Each lane represents an individual mouse of a representative experiment. (b) The ratio phospho-CREB/CREB was not altered in the hippocampus of CB_1 knockout (filled bars) respect wild-type (open bars) mice in basal conditions. (c) No significant difference between genotypes in the total amount of TrkB receptor in the hippocampus. Data are expressed as mean ± SEM of the percentage respect the wild-type group (n = 6–8).

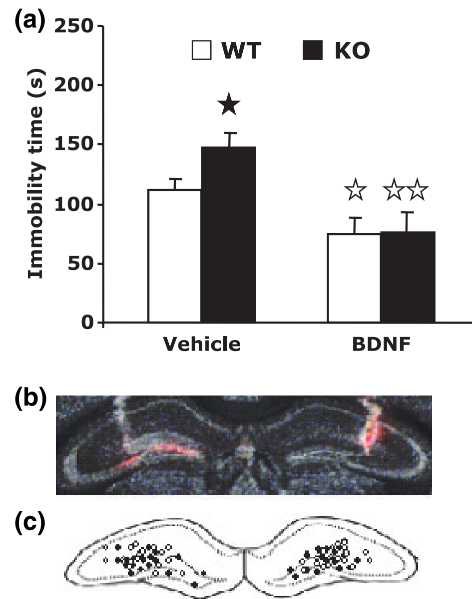

Fig. 4 (a) Despair behavior of wild-type (open bars) and CB_1 knockout (filled bars) mice on the tail suspension test (TST) after local administration of brain derived neurotrophic factor (BDNF) in the hippocampus. Data are expressed as mean ± SEM of the immobility time (n = 9–10). ☆$p < 0.05$, ☆☆$p < 0.01$, when compared with control group of the same genotype. ★$p < 0.05$, comparison between genotypes (one-way ANOVA). (b) Representative coronal section (40 μm) of the mouse brain illustrating the exact location of the cell labeling CM-DiI at the site of infusion in the hippocampus (AP: −1.9; ML: ±1.8, DV: −2.3, expressed in mm) revealed by using a red fluorescent light. (c) Diagram of a hippocampus showing the site of the infusion of vehicle (white dots) and BDNF (black dots).

associated to increased corticosterone serum levels. The hyperactivity of the HPA axis in response to stress reported in this study is in agreement with previous findings (Barna *et al.* 2004; Urigüen *et al.* 2004), and could be due to impairment in the negative feedback regulation of the HPA axis (Cota *et al.* 2007). Accordingly, endogenous cannabinoids inhibit the HPA axis via centrally located CB_1 receptors (Di *et al.* 2003; Patel *et al.* 2004). However, no differences between genotypes were found in the levels of corticosterone under basal conditions, in contrast to a previous study (Urigüen *et al.* 2004). These controversial data should be explained by the different experimental conditions that were considered as baseline in both studies.

Sustained elevations of glucocorticoids have been shown to produce detrimental effects in synaptic plasticity, dendritic morphology (Watanabe *et al.* 1992) and neurogenesis (Warner-Schmidt and Duman 2006), even inducing neuronal death (Sapolsky 2000b) in the hippocampus. In consequence, these hippocampal changes in synaptic connectivity induced by stress might reduce the inhibitory control that this structure exert on the HPA axis, leading to a positive feedback process with pathological consequences that could induce hippocampal volume reduction and cognitive impairments (Radley and Morrison 2005; Bremner 2006). Then, the hyperactivity of the HPA axis in CB_1 knockout mice could be related to the plasticity impairment observed in these mutants.

Brain derived neurotrophic factor modulates plasticity and survival of adult neurons and glia (Huang and Reichardt 2001), and directly modifies the HPA axis activity at the hypothalamic level (Givalois *et al.* 2004). We describe for the first time a deficit at the protein level of the neurotrophic factor BDNF in the hippocampus of CB_1 knockout mice under basal conditions. Our finding is supported by the recently described reduction of the BDNF mRNA expression on the CA3 subfield of the hippocampus of CB_1 mutant mice (Steiner *et al.* 2007). An increase in the hippocampal BDNF content in both genotypes was observed in response to stress (TST exposure), in agreement with the rapid induction of BDNF expression previously reported after short stress exposure (Marmigere *et al.* 2003). However, BDNF levels were significantly lower in mutants than in wild-type mice in response to stress, highlighting the BDNF impairment in mice lacking CB_1 receptor. The BDNF immunoreactivity of coronal sections through the hippocampus revealed that this impairment was homogenously observed in all the regions of the hippocampus of CB_1 mutants. BDNF was not modified in mutant mice in two other brain areas related with stress regulation, frontal cortex and amygdala, revealing an

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anatomical specificity for this neurotrophic impairment, in agreement with the reported vulnerability of the hippocampus to the detrimental effect of stress (McEwen 1999). The neurotrophic deficiency observed in the hippocampus of CB_1 knockout mice seems to be specific for BDNF as no differences were found in basal conditions in the levels of NGF and NT-3, two additional neurotrophic factors. Therefore, BDNF is a specific target for the impairment in plasticity induced by stress in CB_1 knockout mice and this neurotrophic factor seems to be a key mediator in the CB_1 receptor-dependent mechanisms of neuroprotection (Khaspekov *et al.* 2004). The deficit in BDNF mediated plasticity here reported on CB_1 knockout mice could be related to the loss of neurons in the CA1 and CA3 hippocampus regions of aged mice (Bilkei-Gorzo *et al.* 2005) and the increased susceptibility to neurotoxic insults (Marsicano *et al.* 2003) previously described in these mutant mice.

To clarify the mechanisms involved in this BDNF impairment, we have investigated in CB_1 knockout mice the possible changes on BDNF receptor and CREB, a transcription factor directly related to BDNF (Conti *et al.* 2002). CREB represents a central integrator of signaling from several extracellular stimuli that influence neuronal plasticity and survival (Duman 2002). No changes on CREB activity were observed in the hippocampus of CB_1 knockout mice suggesting that the described BDNF impairment is independent of the transcription mechanisms related to CREB. Moreover, BDNF deficit does not produce alterations in the density of TrkB, the specific receptor by which BDNF exerts most of its biological effect (Kaplan and Miller 2000).

To verify the functional role of the hippocampal BDNF impairment in the enhanced despair behavior exhibited by CB_1 knockout mice, the behavioral effects of a local BDNF microinjection in the hippocampus were evaluated. BDNF reduced the immobility time during the TST in both genotypes, and interestingly, completely reversed the increased despair behavior shown by CB_1 knockout mice. This finding confirms the crucial role played by BDNF on the emotional impairment of these mutants. In spite of the relevance of this behavioral finding, the biochemical mechanisms underlying the link between CB_1 receptor and BDNF can not be yet completely elucidated. We hypothesize that the BDNF impairment here reported could be induced by the hyperactivity of the HPA axis in the absence of the inhibitory effect of the CB_1 receptor. There are evidence showing that BDNF expression is decreased by glucocorticoids (Smith *et al.* 1995; Schaaf *et al.* 1998; Jacobsen and Mork 2006). The mechanisms by which steroids exert this control on BDNF expression are not completely elucidated, but it has been described that it could be via activated gluco- and mineralocorticoid receptors (Hansson *et al.* 2000) or inducing sustained extracellular signal-regulated kinase/mitogen-activated protein kinase activation (Yang *et al.* 2004). However, other potential explanations are equally probable. Considering a possible CB_1-dependent activation of the BDNF, the interruption of this signaling cascade in CB_1 knockout mice could first diminish BDNF levels and then the induced plasticity impairment could modify the HPA axis regulation in these mutants.

In summary, our results provide evidence for neurotrophic and neuroendocrine changes in CB_1 knockout mice that could explain the neurobiological substrate underlying the enhanced stress response and despair behavior exhibited by these mutants. We demonstrate that the lack of CB_1 receptor originates a deficiency in the feedback regulation of the HPA axis, which seems to be related to the decreased BDNF levels observed in the hippocampus of CB_1 knockout mice. The hippocampal BDNF impairment has an essential role in the altered stress response of CB_1 mutants as demonstrated the complete abolition of the increased despair behavior by the hippocampal administration of BDNF. Taken together, our data reveal the neurotrophic and neuroendocrine mechanisms involved in the control of emotional responses by the endocannabinoid system, and further emphasize the relevance of this system as a possible therapeutic target for the management of stress-related mood disorders.

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