

BDNF impairment in the hippocampus is related to enhanced despair behavior in CB₁ knockout mice

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Abbreviations: brain derived neurotrophic factor (BDNF), cAMP responsive element-binding (CREB), hypothalamic-pituitary-adrenal (HPA), recombinant human BDNF (rhBDNF), tail suspension test (TST), tyrosine kinase receptor (TrkB)

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 CB₁ receptor alters neuronal plasticity and originates an anxiety-like phenotype in mice. In the present study, CB₁ 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 CB₁ 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 since no differences were found in the levels of NGF and NT-3, two additional neurotrophic factors. Moreover, BDNF impairment is not related to the activity of its specific receptor TrkB or the activity of the transcription factor CREB. These results suggest that the lack of CB₁ receptor originates an enhanced response to stress and neuronal plasticity by decreasing BDNF levels in the hippocampus that lead to impairment in the responses to emotional disturbances.

Keywords: stress, CB₁ cannabinoid receptor, corticosterone, BDNF, CREB, TrkB

Running title: BDNF impairment in CB₁ knockout mice

Introduction

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 due to 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 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 evidence 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 CB₁ receptor, the most abundant cannabinoid receptor in the brain, is expressed in all of the main brain structures involved in stress-related behaviours 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 CB₁ 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 CB₁ 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 CB₁ 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 CB₁ knockout mice and wild-type littermates 8-12 weeks old at the beginning of the experiments. The generation of mice lacking CB₁ cannabinoid receptor was described previously (Ledent et al, 1999). In order 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 CB₁ gene at each generation. Beginning with the 30th generation backcrossing mice, heterozygote-heterozygote mating of CB₁ 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-h light/dark cycle (light between 08:00 and 20:00 h) 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 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgCl₂ in distilled water, all the components provided by Scharlau Chemie, Paris, France). The

cell labelling CellTrackerTM CM-DiI was supplied by Molecular ProbesTM (Eugene, Oregon, 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 one hour 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 CB₁ knockout mice and wild-type littermates which were killed immediately after taking them from their home cage.

BDNF hippocampal microinjection

rhBDNF was locally administered into the hippocampus (0.25 $\mu\text{g}/\text{side}$). Mice were first anaesthetized 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, U.S.A.). The coordinates (AP: -1.9; ML: \pm 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 $\mu\text{L}/\text{side}$ of vehicle or rhBDNF and 0.2 $\mu\text{L}/\text{side}$ 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. In order to evaluate the effect of hippocampal rhBDNF microinjection,

TST was performed, as described previously, three days after the surgery. After TST, mice were killed and the brains were 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-Dil at the site of infusion, although small amounts of CM-Dil 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 mM NaCl; 20 mM Tris-HCl, pH 8.0; 1% NP-40; 10% glycerol; 1 mM sodium vanadate; 5 mM sodium pyrophosphate; 100 mM NaF; 40 mM glycerol phosphate; 1mM PMSF; 0.15 μM aprotinine; 11 μM leupeptine; 1.5 μM pepstatine) in order 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 16000 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 anaesthetised by intraperitoneal (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 (PFA) in 0.1M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$

buffer (PB), pH 7.5, delivered with a peristaltic pump at 19 mL/min for 5 min. Brains were removed and postfixed overnight at 4 °C in the same fixative solution. Brain free-floating sections (30µm) through the hippocampus were obtained with a vibratome (Leica, France) and kept in a cryoprotection solution containing 30% ethylene glycol, 30% glycerol and 0.1M PB 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 ¹²⁵I 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[®], Madison, WI, USA) was used to quantify the levels of BDNF protein. Prior to each assay, samples were diluted and acid-treated in order 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 1M HCl/50 µL sample and after 15 min of incubation at room temperature, samples were neutralized with the same amount of NaOH 1M. 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 NGF and NT-3 levels by using specific immunoassays (Promega Corporation[®], Madison, WI, USA). Data were calculated as percentage of respective control and were expressed as mean \pm SEM.

BDNF immunohistochemistry

Free-floating sections were washed in 0.25 M Tris buffered saline (TBS), incubated in TBS containing 3% H₂O₂ 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 room temperature. Sections were incubated with the rabbit anti-BDNF antibody (Santa Cruz Biotechnology Inc, 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 hr. After being washed, the sections were incubated for 90 min in avidin-biotin-peroxidase complex (ABC) solution (final dilution, 1:50; Vector Laboratories, Peterborough, UK). Sections were then washed in TBS and in TB (0.25 M TRIS, pH 7.5), placed in a solution of TB containing 0.1% 3,3'-diaminobenzidine (DAB; 30mg/100 mL), and developed by H₂O₂ 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

CREB, phospho-CREB and TrkB levels were quantified by western blotting. Equal amounts of lysates (40-50 μ g per lane) were separated by SDS-PAGE (12.5%) before electrophoretic transfer onto PVDF membrane (CREB detection) or nitrocellulose membrane (phospho-CREB and TrkB detection) (Bio-Rad, Spain). Membranes were blocked for 1 hour at room temperature in Tris-buffered saline (TBS) (100 mM NaCl, 10 mM Tris, pH7.4) with 0.1% Tween 20, 1 mM sodium orthovanadate, 1 mM NaF (0.5M) and 5% nonfat milk (CREB detection) or 3% nonfat milk (phospho-CREB

detection). Afterwards, membranes were incubated overnight with the primary antibodies (CREB, 1:800, Upstate; 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; diluted 1:60000, 1 hour at room temperature) and visualized by enhanced chemiluminescence detection (ECL AdvanceTM, Amersham Biosciences). 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). 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 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 \pm 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 CB₁ knockout mice

The exposure to TST induced a higher immobility time in CB₁ knockout mice than in wild-type littermates ($F_{(1, 13)} = 9.412$, $p < 0.01$), revealing an increased despair behavior in mice lacking CB₁ receptor (Fig 1A).

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 CB₁ knockout mice ($F_{(1, 14)} = 54.970$, $p < 0.001$). One-way ANOVA calculated for genotype effect showed higher levels of corticosterone in CB₁ 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 CB₁ knockout mice

BDNF protein quantification (ELISA)

CB₁ knockout mice showed decreased BDNF levels in the hippocampus at basal conditions (wild-type: 651 ± 51 ng BDNF/g wet weight; CB₁ 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 CB₁ knockout mice ($F_{(1, 15)} = 84.631$, $p < 0.001$). Comparisons between genotypes revealed lower BDNF levels in CB₁ 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 CB₁ knockout mice.

The neurotrophic deficiency in the hippocampus of CB₁ knockout mice seems to be specific for BDNF since 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

CB₁ 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.

CREB and TrkB quantification

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

Reversal of CB₁ 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 CB₁ 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 CB₁ knockout mice ($F_{(1, 18)} = 11.877$, $p < 0.01$). Comparisons between genotypes showed a significant increase in the immobility time in vehicle-treated CB₁ 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 CB₁ knockout mice. This neurotrophic factor plays a key role in the enhanced response to stress observed in mutant mice since BDNF administration in the hippocampus completely reversed the increased despair behavior exhibited by CB₁ knockout mice in the tail suspension test.

Mice lacking CB₁ 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 CB₁ 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 CB₁ 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 CB₁ knockout mice when exposed to a single TST was 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 CB₁ 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 CB₁ knockout mice could be related to the plasticity impairment observed in these mutants.

BDNF 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 CB₁ knockout mice under basal conditions. Our finding is supported by the recently described reduction of the BDNF mRNA expression on the CA3 sub-field of the hippocampus of CB₁ 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 CB₁ receptor. The BDNF immunoreactivity of coronal sections through the hippocampus revealed that this impairment was homogeneously observed in all the

regions of the hippocampus of CB₁ mutants. BDNF was not modified in mutant mice in two other brain areas related with stress regulation, frontal cortex and amygdala, revealing an 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 CB₁ knockout mice seems to be specific for BDNF since 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 CB₁ knockout mice and this neurotrophic factor seems to be a key mediator in the CB₁ receptor-dependent mechanisms of neuroprotection (Khaspekov et al, 2004). The deficit in BDNF mediated plasticity here reported on CB₁ 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.

In order to clarify the mechanisms involved in this BDNF impairment, we have investigated in CB₁ 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 CB₁ 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).

In order to verify the functional role of the hippocampal BDNF impairment in the enhanced despair behavior exhibited by CB₁ knockout mice, the behavioral effects of a

local BDNF microinjection in the hippocampus was evaluated. BDNF reduced the immobility time during the TST in both genotypes, and interestingly, completely reversed the increased despair behavior shown by CB₁ 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 CB₁ 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 CB₁ 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 CB₁-dependent activation of the BDNF, the interruption of this signaling cascade in CB₁ 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 CB₁ 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 CB₁ 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 CB₁ knockout mice. The hippocampal BDNF impairment has an essential role in the altered stress response of CB₁ 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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References

- Barna I., Zelena D., Arszovszki A.C., Ledent C. (2004) The role of endogenous cannabinoids in the hypothalamo-pituitary-adrenal axis regulation: in vivo and in vitro studies in CB₁ receptor knockout mice. *Life Sci* **75**, 2959-2970.
- Bilkei-Gorzo A., Racz I., Valverde O., Otto M., Michel K., Sastre M., Zimmer A. (2005) Early age-related cognitive impairment in mice lacking cannabinoid CB₁ receptors. *Proc Natl Acad Sci U S A* **102**, 15670-15675.
- Bremner J.D. (2006) Stress and brain atrophy. *CNS Neurol Disord Drug Targets* **5**, 503-512.
- Conti A.C., Cryan J.F., Dalvi A., Lucki I., Blendy J.A. (2002) cAMP response element-binding protein is essential for the upregulation of brain-derived neurotrophic factor transcription, but not the behavioral or neuroendocrine responses to antidepressant drugs. *J Neurosci* **22**, 3262-3268.
- Cota D., Steiner M.A., Marsicano G., Cervino C., Herman J.P., Grubler Y., Stalla J., Pasquali R., Lutz B., Stalla G.K., Pagotto U. (2007) Requirement of cannabinoid receptor type 1 for the basal modulation of hypothalamic-pituitary-adrenal axis function. *Endocrinology* **148**, 1574-1581.
- Di S., Malcher-Lopes R., Halmos K.C., Tasker J.G. (2003) Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. *J Neurosci* **23**, 4850-4857.
- Duman R.S. (2002) Pathophysiology of depression: the concept of synaptic plasticity. *Eur Psychiatry* **17**, 306-310.
- Duman R.S., Monteggia L.M. (2006) A Neurotrophic Model for Stress-Related Mood Disorders. *Biol Psychiatry* **59**, 1116-1127.

- Fride E., Suris R., Weidenfeld J., Mechoulam R. (2005) Differential response to acute and repeated stress in cannabinoid CB₁ receptor knockout newborn and adult mice. *Behav Pharmacol* **16**, 431-440.
- Givalois L., Naert G., Rage F., Ixart G., Arancibia S., Tapia-Arancibia L. (2004) A single brain-derived neurotrophic factor injection modifies hypothalamo-pituitary-adrenocortical axis activity in adult male rats. *Mol Cell Neurosci* **27**, 280-295.
- Hansson A.C., Cintra A., Belluardo N., Sommer W., Bhatnagar M., Bader M., Ganten D., Fuxe K. (2000) Gluco- and mineralocorticoid receptor-mediated regulation of neurotrophic factor gene expression in the dorsal hippocampus and the neocortex of the rat. *Eur J Neurosci* **12**, 2918–2934.
- Herman J.P., Ostrander M.M., Mueller N.K., Figueiredo H. (2005) Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Prog Neuropsychopharmacol Biol Psychiatry* **29**, 1201-1213.
- Hill M.N., Gorzalka B.B. (2005) Is there a role for the endocannabinoid system in the etiology and treatment of melancholic depression? *Behav Pharmacol* **16**, 333-352.
- Huang E.J., Reichardt L.F. (2001) Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* **24**, 677-736.
- Jacobsen J.P., Mork A. (2006) Chronic corticosterone decreases brain-derived neurotrophic factor (BDNF) mRNA and protein in the hippocampus, but not in the frontal cortex, of the rat. *Brain Res* **1110**, 221-225.
- Jin K., Xie L., Kim S.H., Parmentier-Batteur S., Sun Y., Mao X.O., Childs J., Greenberg D.A. (2004) Defective adult neurogenesis in CB₁ cannabinoid receptor knockout mice. *Mol Pharmacol* **66**, 204-208.
- Kaplan D.R., Miller F.D. (2000) Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol* **10**, 381-391.

- Khaspekov L.G., Brenz Verca M.S., Frumkina L.E., Hermann H., Marsicano G., Lutz B. (2004) Involvement of brain-derived neurotrophic factor in cannabinoid receptor-dependent protection against excitotoxicity. *Eur J Neurosci* **19**, 1691-1698.
- Ledent C., Valverde O., Cossu G. et al (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB₁ receptor knockout mice. *Science* **283**, 401-404.
- Lindvall O., Kokaia Z., Bengzon J., Elmer E., Kokaia M. (1994) Neurotrophins and brain insults. *Trends Neurosci* **17**, 490-496.
- Marmigere F., Givalois L., Rage F., Arancibia S., Tapia-Arancibia L. (2003) Rapid induction of BDNF expression in the hippocampus during immobilization stress challenge in adult rats. *Hippocampus* **13**, 646-655.
- Marsicano G., Goodenough S., Monory K. et al (2003) CB₁ cannabinoid receptors and on-demand defense against excitotoxicity. *Science* **302**, 84-88.
- Martín M., Ledent C., Parmentier M., Maldonado R., Valverde O. (2002) Involvement of CB₁ cannabinoid receptors in emotional behavior. *Psychopharmacology (Berl)* **159**, 379-387.
- Matsuda L.A., Bonner T.I., Lolait S.J. (1993) Localization of cannabinoid receptor mRNA in rat brain. *J Comp Neurol* **327**, 535-550.
- McEwen B.S. (1999) Stress and hippocampal plasticity. *Annu Rev Neurosci* **22**, 105-122.
- Nestler E.J., Gould E., Manji H. et al (2002) Preclinical models: status of basic research in depression. *Biol Psychiatry* **52**, 503-528.
- Okragly A.J., Haak-Frendscho M. (1997) An acid-treatment method for the enhanced detection of GDNF in biological samples. *Exp Neurol* **145**, 592-596.

- Patel S., Roelke C.T., Rademacher D.J., Cullinan W.E., Hillard C.J. (2004) Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. *Endocrinology* **145**, 5431-5438.
- Paxinos, G., Franklin, K.B.J. (1997) *The Mouse Brain in Stereotaxic Coordinates*. Academic Press, San Diego.
- Radley J.J., Morrison J.H. (2005) Repeated stress and structural plasticity in the brain. *Ageing Res Rev* **4**, 271-287.
- Sapolsky R.M. (2000a) Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch Gen Psychiatry* **57**, 925-935.
- Sapolsky R.M. (2000b) The possibility of neurotoxicity in the hippocampus in major depression: a primer on neuron death. *Biol Psychiatry*, **48**, 755-765.
- Schaaf M.J., de Jong J., de Kloet E.R., Vreugdenhil E. (1998) Downregulation of BDNF mRNA and protein in the rat hippocampus by corticosterone. *Brain Res* **813**, 112-120.
- Smith M.A., Makino S., Kvetnansky R., Post R.M. (1995) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J Neurosci* **15**:1768-1777.
- Steiner M.A., Wanisch K., Monory K., Marsicano G., Borroni E., Bachli H., Holsboer F., Lutz B., Wotjak C.T. (2007) Impaired cannabinoid receptor type 1 signaling interferes with stress-coping behavior in mice. *Pharmacogenomics J*, **Aug 7**
- Steru L., Chermat R., Thierry B., Simon P. (1985) The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl)* **85**, 367-370.
- Urigüen L., Pérez-Rial S., Ledent C., Palomo T., Manzanares J. (2004) Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB1 receptors. *Neuropharmacology* **46**, 966-973.

Valverde O. (2005) Participation of the cannabinoid system in the regulation of emotional-like behavior. *Curr Pharm Des* **11**, 3421-3429.

Warner-Schmidt J.L., Duman R.S. (2006) Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment. *Hippocampus* **16**, 239-249.

Watanabe Y., Gould E., McEwen B.S. (1992) Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. *Brain Res* **588**, 341-345.

Yang C.H., Huang C.C., Hsu K.S. (2004) Behavioral stress modifies hippocampal synaptic plasticity through corticosterone-induced sustained extracellular signal-regulated kinase/mitogen-activated protein kinase activation. *J Neurosci* **24**, 11029-11034.

Figure 1. Enhanced response to stress in CB₁ knockout mice. (A) Increased despair behavior of CB₁ knockout (filled bars) compared to wild-type (open bars) mice on the TST. (B) Serum corticosterone levels were increased in CB₁ knockout mice one hour after exposure to the TST compared to wild-type mice but not in basal conditions. Data are expressed as mean \pm SEM (n = 8-10). ☆☆☆ p < 0.001, when compared to basal group of the same genotype. ★ p < 0.05, ★★ p < 0.01, comparison between genotypes (one-way ANOVA).

Figure 2. BDNF impairment in the hippocampus of CB₁ knockout mice. (A) Decreased BDNF levels measured by ELISA in the hippocampus of CB₁ knockout (filled bars) compared to 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 CB₁ knockout (lower panel) mice in basal conditions. Right panels illustrate higher-magnification pictures of the CA₃ region of the hippocampus. Scale bar represents 100 μ m in the left panels and 50 μ m in right panels. DG: dentate gyrus. (C) Measurement of the BDNF levels by ELISA in the frontal cortex (prelimbic and motor cortex) and amygdala revealed no difference between genotypes in basal conditions. (D) Neurotrophic deficiency in the hippocampus of CB₁ knockout mice seems to be specific for BDNF since there is not difference between genotypes in the NGF and NT-3 protein contents measured by ELISA. Data are expressed as mean \pm SEM of the percentage of neurotrophic factors respect the wild-type control group (n = 8-10). ☆☆ p < 0.01, when compared to control group of the same genotype (one-way ANOVA). ★ p < 0.05, ★★ p < 0.01, comparison between genotypes (one-way ANOVA).

Figure 3. BDNF basal impairment is independent of the transcription factor CREB activity or the density of the specific 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 CB₁ 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 \pm SEM of the percentage respect the wild-type group (n = 6-8).

Figure 4. (A) Despair behavior of wild-type (open bars) and CB₁ knockout (filled bars) mice on the TST after local administration of BDNF in the hippocampus. Data are expressed as mean \pm SEM of the immobility time (n = 9-10). ☆ p < 0.05, ☆☆ p < 0.01, when compared to control group of the same genotype. ★ p < 0.05, comparison between genotypes (one-way ANOVA). (B) Representative coronal section (40 μ m) 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: \pm 1.8, DV: -2.3) 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).