Depression-resistant endophenotype in mice overexpressing cannabinoid CB2 receptors

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Abstract

Background: The present study evaluated the role of the CB2r in the regulation of depressive-like behaviors. To this aim, transgenic mice overexpressing the CB2r (CB2xP) were challenged against different types of acute and chronic experimental paradigms to evaluate its response to depressive-like behaviors.

Methods: Tail suspension (TST), novelty suppressed feeding (NSFT) and unpredictable chronic mild stress tests (CMS) were carried out in CB2xP. Furthermore, acute and chronic antidepressant-like effects of the CB2r antagonist AM630 were evaluated (forced swimming test (FST) and CMS, respectively) in WT mice.

Results: Overexpression of the CB2r resulted in decreased depressive-like behaviors in TST and NSFT. CMS failed to produce any alteration in the CB2xP in TST and sucrose consumption. In addition, no changes were observed in BDNF gene and protein expressions in stressed-CB2xP mice. Interestingly, the administration of AM630 (1 and 3 mg/kg, i.p.) decreased the emotional-behavior response to acute anxiety-stimuli (FST) in WT mice. Repeated administration of AM630 for 4 weeks (1 mg/kg; twice a day; i.p.) blocked the effects of CMS on TST, sucrose intake, BDNF gene and protein expressions.

Conclusions: Taken together, these results suggest that increased expression of the CB2r significantly reduced depressive-related behaviors and point out the CB2r as a new potential key target in the treatment of depression related disorders.

Key words: cannabinoid CB2 receptor, depression, antidepressant, brain-derived neurotrophic factor, unpredictable chronic mild stress, behavior, transgenic mice

1. INTRODUCTION

The limited efficacy of current antidepressant treatments requires the development of alternative drugs. Recent pharmacological and genetic findings revealed the endocannabinoid system as a target closely related with the regulation of mood disorders. In fact, cannabinoid CB1 antagonists, SR141716A (rimonabant) and AM251, have presented antidepressant-like effects in animals models of depression (1-4). In addition, 5-HT, NA and dopamine levels in the prefrontal cortex increased after the administration of rimonabant (1;2;4;5). However, paradoxically rimonabant has been linked to increased risk of anxiety, depression and suicidal thoughts (6;7) in the treatment of depressive disorders in humans. Cannabinoid CB1 agonists and fatty acid amide hydrolase (FAAH) inhibitors induce antidepressant-like effects in the forced swimming test in rats (8) providing further support of a pivotal role of the endocannabinoid system in the pathogenesis of depression. Chronic treatment with these drugs promoted neurogenesis in hippocampus and enhanced central serotoninergic and noradrenergic transmission (9). In addition, different animal models of depression revealed significant increases in CB1r density and function at the prefrontal cortex (10;11) that may be reversed by chronic fluoxetine treatment (10). Clinically, significant up-regulation of CB1r density and CB1r-stimulated G-protein activation was found in the prefrontal cortex of depressive suicide victims (12).

Initially, CB2r was identified in the brain only under pathological conditions such as senile plaques in Alzheimer's disease (13), in activated microglial cells/macrophages of multiple sclerosis (24), amyotrophic lateral sclerosis spinal cord (14) and near to tumors (15;16). Recently, CB2r were found under normal conditions in the brainstem of rat, mouse and ferret (17). Further studies in the rat identified a wide distribution of CB2r in different brain areas including spinal nucleus, hippocampus, olfactory nucleus, cerebral cortex, amygdala, striatum, thalamus and cerebellum (18;19). The presence of CB2r in these areas suggests its potential role in the regulation of anxiety and depression.

Interestingly, a reduction of CB2r in striatum, midbrain and hippocampus was reported in animal models of depression (20). Conversely, intracerebroventricular microinjection of cannabinoid CB2 antisense oligonucleotide induced anxiolytic-like effects (21). In addition, an association between cannabinoid CB2r polymorphism Q63R was also detected in Japanese depressed and alcoholic subjects (20).

The responses of transgenic mice overexpressing this receptor (CB2xP) to acute and chronic anxiogenic-like stimuli were studied. The acute (the forced swimming test) and chronic (CMS

procedure) effects of AM630 were evaluated. Furthermore, the influence of CB2r on hippocampal neurogenesis was examined by studying BDNF expression in CB2xP and AM630-treated WT mice exposed to CMS.

2. MATERIALS AND METHODS

2.1. Animals

Male mice overexpressing CB2r (CB2xP) made in our laboratory as described previously (33) and their corresponding littermates (WT) mice (age 2-3 months, 25-35 g) were used in all experiments. All animals were maintained under controlled temperature (23±2ºC) and light (light-dark cycle from 8.00 to 20.00 hours). All studies were performed in compliance with the Royal Decree 223/1998 of 14 March (BOE. 8 18) and the Ministerial Order of 13 October 1989 (BOE 18) as well as with the European Council Directive of 24 November 1986 (86/609/EEC).

2.2. Drugs

AM630 (6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone) was obtained from Tocris (Biogen, Madrid, Spain) and dissolved in DMSO:Tween 80:distilled water (1:1:8) immediately use (22;23). In acute experiments, drug was administered at 1 and 3 mg/kg (i.p., 0.3 ml) 30 minutes before the corresponding experimental test. At the end of the 4-week of CMS, mice were assigned to different groups, so the initial coat state and body weight were equivalent in all groups. Drug was given twice daily (9.00 and 18.00) at 1 mg/kg (i.p., 0.3 ml) during 4 weeks.

2.3. Behavioral analyses

2.3.1. Tail suspension test

Mice were individually suspended by the tail on the edge of a lever above the table top (distance from the floor was 35 cm) by using adhesive tape placed approximately 1-2 cm from the tip of the tail (24). The time of immobility was measured during a period of 6 minutes. In this situation, mice develop escape-oriented behaviors interspersed with temporally increasing bouts of immobility.

2.3.2. Novelty suppressed feeding test

The testing apparatus consisted of a transparent methacrylate square cage 40 x 40 x 50 cm (37). At the time of testing, a single pellet of food was placed on a white paper platform in the center of the cage. Mice fasted for 24 hours, were individually placed in the corner of the apparatus. The time of latency to initiate consumption of the pellet was recorded to a ceiling of 5 minutes. Once the mice started to eat, the total amount of food pellet (g) was measured during a period of 5 minutes.

2.3.3. Forced swimming test

The FST has been used as a model predictive of antidepressant action (38). Briefly, each mouse was placed for 15 min in vertical Plexiglas cylinder (height 25 cm diameter 18 cm) containing water to a depth of 15 cm at 25±1 °C (25). After 24 h, animals were placed again into the cylinder and the duration of the immobility was measured during a period of 5 min. Only active swimming, not floating movements, was taken into account for immobility measurement.

2.3.4. Chronic unpredictable mild stress

Mice were exposed to CMS during a period of 7-8 weeks (26;27). Mice were subjected several times per day to one or more of the following stressors: wet cage, food deprivation, restraint stress, period of stroboscopic illumination (150 flashes/min), inversion of light/dark cycle, tilted cage (45º) and strong noise (90-105 db). All the stressors and/or sequences were applied at different times point to avoid habituation and to provide an unpredictable feature to the stressors (Table 1).

2.3.5. Sucrose consumption test

Sucrose intake (1% sucrose solution) was measured after 18 hours of food and water deprivation (28). Consumption of sucrose solution was estimated simultaneously in control and experimental groups by comparing bottle weight before and after the 1-h window. The sucrose intake was expressed as mg sucrose/g body weight).

2.4 Analysis of BDNF gene expression

Brain sections were cut at 500 µm at different levels containing the regions of interest according to Paxinos and Franklin (29), mounted onto slides and stored at −80 °C. Sections were dissected following the method described by Palkovits (30). Total RNA was obtained from brain punches using Biozol® Total RNA extraction reagent (Bioflux, Inilab, Madrid, Spain). After DNAse digestion, the reverse transcription was carried out following the instructions of the manufacturer (Epicentre, Tech. Corp., Madison, Wisconsin). BDNF gene expression was measured by using Taqman Gene Expression assay (Mm00432069_m1) (Applied Biosystems, Madrid, Spain) as a double-stranded DNA-specific fluorescent dye and performed on the AbbiPrism 7700 Real Time Cycler (Applied Biosystems, Madrid, Spain). The reference gene used was 18S rRNA, detected using Taqman

ribosomal RNA control reagents. Briefly, data for each target gene were normalized to the endogenous reference gene, and the fold change in target gene abundance was determined using the 2-ΔΔCt method (31).

2.5. Immunohistochemistry

CB2xP and WT mice (n=3-5/group) were anesthetized with ketamine/xylacine (2:1 v/v, 0.2 ml), and intracardially perfused with 200 ml of 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). Brains were dissected, postfixed in the same fixative solution overnight at 4 ºC, frozen and cut into coronal 50 µm sections using a vibratome. For CB2r, floating sections were pre-incubated with 50 mM sodium citrate pH 9, for 30 min at 80 ºC, washed three times with phosphate-buffered saline (PBS; 0.1 M, pH 7.3), and then incubated with 1% hydrogen peroxide in PBS for 20 min at room temperature to inhibit endogenous peroxidase, washed three times with PBS, incubated for 1 h in 10% normal goat serum (NGS) in PBS and 0.3% triton X-100, at room temperature. Then, sections were incubated in primary CB2 antibody obtained from Cayman Chemicals (MI, USA), diluted 1:500 in PBS + 0.3% triton X-100, overnight at room temperature, rinsed, incubated for 1 h at room temperature in 1:500 dilution of biotinylated goat anti-rabbit secondary antibody (Vector, Burlingame, CA, USA) in PBS + 0.3% triton X-100, rinsed, incubated in extravidin-peroxidase (Sigma-Aldrich, Madrid, Spain) diluted 1:2000 in PBS + 0.2% triton X-100 for 1 h at room temperature, rinsed, and then incubated in a solution containing 0.05% diaminobenzidine-niquel (DAB-Ni) (Sigma-Aldrich, Madrid, Spain) and 0.003% hydrogen peroxide for color deposition. Sections were mounted on coated slides, dehydrated, cover slipped, viewed and photographed using Zeiss and Leitz microscope and a Nikon digital camera, and images were edited using photoshop (vCS3; Adobe systems) and quantified using Image J software (National Institutes of Health, USA) after background subtraction.

For BDNF, floating sections were washed three times with PBS, and then incubated with 1% hydrogen peroxide in methanol:PBS (1:1) for 15 min at room temperature to inhibit endogenous peroxidase, washed three times with PBS + 0.2% triton X-100 (PBS-T), incubated for 1 h in 10% NGS in PBS-T at room temperature. Then, sections were rinsed and incubated in primary BDNF antibody obtained from Chemicon (Temecula, CA, USA), diluted 1:100 in PBS-T, overnight at 4 ºC, rinsed, incubated for 1.5 h at room temperature in 1:200 dilution of biotinylated goat anti-rabbit secondary antibody (Vector, Burlingame, CA, USA) in PBS-T, rinsed, incubated in extravidin-peroxidase (Sigma-

Aldrich, Madrid, Spain) diluted 1:500 in PBS-T for 1.5 h at room temperature, rinsed, and then incubated in a solution containing 0.05% diaminobenzidine (Sigma-Aldrich, Madrid, Spain) and 0.003% hydrogen peroxide for color deposition. Sections were mounted and analyzed as mentioned before.

2.6. Statistical analyses

Statistical analyses were performed using the Student t-test when comparing two groups and one-way or two-way analysis of variance followed by the Student Newman Keul's test when comparing three or four groups. Differences were considered significant if the probability of error was less than 5%. Data are presented as mean ± S.E.M. SigmaStat 3.1 software was used for all statistical analyses.

3. RESULTS

3.1 Characterization of CB2r expression

3.1.1 Analysis of CB2r gene expression

The analyses of the different brain punches in WT mice by Rt-PCR revealed the presence of CB2r gene expression in almost all the nuclei examined. The results were expressed considering the CPu as 100% (arbitrarily). In this manner, CB2r gene expression resulted significantly higher in Acc (137%), Amy (107%), VMN (90%), Sn (224.62%) and MnR (84%) compared to CPu (n= 6-7/group) (see Table 2 and Figure 1A).

CB2xP mice presented significant increased CB2r mRNA levels in all the regions analyzed compared to WT mice (Student t-test, CPu t= -3.047, p<0.011, 11 df (150%); Acc t= -4.590, p= 0.001, 9 df (180%); Cg t=-6.125, p<0.001, 9 df (199%); Amy t= -1.573, p= 0.05, 9 df (64%); Hipp t= -2.464, p= 0.027, 14 df (158%); VMN t= -2.863, p<0.001, 9 df (126%); ARC t= -2.184, p= 0.05, 9 df (157%); SN t= -3.36, p= 0.006, 11 df (278%); VTA t= 3.876, p= 0.003, 10 df; DR t= 2.548, p=0.031, 9 df (50%); MnR t= 2.987, p= 0.014, 10 df) (n= 6-7/group) (Figure 1A).

3.1.2 CB2r protein expression in the hippocampus

The results revealed increased expression of CB2 protein in different fields of hippocampus (CA2, CA3 and DG) in CB2xP mice compared to WT mice (Student t-test CA2 t= 5.451, p= 0.002, 6 df ; CA3 t= 4.278, p= 0.005, 6 df; DG t= 3.937, p= 0.008, 6 df) (n= 4/group). No differences were detected in CA1 field (Student t-test t= 1.642, p= 0.152, 6 df) (n= 4/group) (Figure 1B and 1C).

3.2 Behavioral analyses

The effects of CB2xP and WT mice to acute and chronic anxiogenic-like stimuli were evaluated by using TST, NSFT and CMS tests.

CB2xP mice showed significantly less time of immobility compared to WT mice during the TST (Student t-test, t= 5.723, p<0.001, 20 df) (n=12-14/group) (Figure 2A).

Accordingly, CB2xP mice presented significantly lower time of latency (Student t-test, t= 2.463, p= 0.023, 20 df) and displayed pronounced increase in consumption of food pellets (g) compared to WT mice during the NSFT (Student t-test, t= -3.711, p= 0.001, 21 df) (n=11-12/group) (Figure 2B).

In CMS, depressive-like behaviors were evaluated at different time points by using the TST (5 week) and sucrose intake tests (7 week). In the TST, stressed-WT mice present significant higher time of immobility compared with non-stressed-WT mice (Student t-test, t= -2.664, p= 0.011, 20 df) (n= 12/group) (Figure 2 C1, left panel). In contrast, no differences were observed between stressed and non-stressed-CB2xP groups (Student t-test, t= 0.644, p= 0.526, 21 df) (n= 12/group) (Figure 2 C1, right panel).

At the end of 7-weeks of CMS, stressed-WT mice presented significant reduction of sucrose intake (mg sucrose/g bw) compared with non-stressed-WT mice (Student t-test, t=3.151, p=0.025, 5 df) (n=12/group) (Figure 2 C2, left panel). Interestingly, CMS failed to produce any alteration in the sucrose intake of CB2xP mice (Student t-test, t= 0.365, p= 0.721, 13 df) (n= 12/group) (Figure 2 C2, right panel).

3.3 Analysis of BDNF in CB2xP and WT mice exposed to CMS

The expression of BDNF in the hippocampus, especially at the dentate gyrus (DG), is known to be down regulated in response to chronic stress (45). In the present study, hippocampus BDNF gene expression of CB2xP and WT mice exposed to the CMS was measured by real-time PCR. As expected, stressed-WT mice presented significant reduction of BDNF gene expression compared with non-stressed-WT mice. In contrast, CMS failed to produce any alteration in BDNF gene expression of stressed-CB2xP mice. Interestingly, CB2xP mice showed significant basal higher BDNF mRNA levels compared to WT mice (two way ANOVA followed by Student Newman Keul's, genotype $F_{(1,26)}$= 19.033, $p<0.001$; stress $F_{(1,26)}$= 1.520, p= 0.230, genotype x stress $F_{(1,26)}$= 0.299) (n= 5-7/group) (Figure 3 A1).

BDNF protein expression was significantly reduced in the DG of stressed-WT compared to non-stressed-WT mice. In contrast, no alterations were found in CB2xP mice (two way ANOVA followed by Student Newman Keul's, genotype $F_{(1,32)}$= 4.192, p= 0.050; stress $F_{(1,32)}$= 3.355, p= 0.077, genotype x stress $F_{(1,32)}$= 7.381, p= 0.011) (n= 5-6/group) (Figure 3 A2 and A3).

3.4 Acute and chronic effects of cannabinoid CB2 r antagonist

The effects of acute administration of AM630 on the response to anxiogenic-like stimuli were evaluated in CB2xP and WT mice by using the FST. The results revealed that AM630 increases

significantly the time of immobility in WT mice at 1 and 3 mg/kg (one way ANOVA $F_{(2,18)}$= 15.506, $p<0.001$) (n= 7-9 per group) (Figure 4A, left panels). In contrast, this drug failed to produce any change in CB2xP mice (one way ANOVA $F_{(2,12)}$= 0.120, p= 0.888) (n= 6-8/group) (Figure 4A, right panels).

In addition, the effects of chronic administration of AM630 on the response to anxiogenic-like stimuli were evaluated in WT mice exposed to CMS. Before administration of AM630, depressive-like behaviors were corroborated at different time points during CMS by using the TST (3rd week) and sucrose intake (4th week). The results revealed significant increased time of immobility in stressed-WT compared with non-stressed-WT mice (Student t-test, t= -2.664, p= 0.011, 38 df) (n= 24/group) (Figure 4 B1). In addition, sucrose intake was significantly reduced in stressed-WT (Student t-test, t= 4.766, p= 0.009, 4 df) (n= 24/group) (Figure 4 B2). These results support that the stressful manipulations carried out during CMS are indeed causing depressive-like behaviors.

Once CMS was established, the chronic treatment with AM630 started. On weeks 3 and 4 after the initiation of the treatment, depressive-like behaviors were evaluated by using TST and sucrose intake test, respectively. As expected, vehicle stressed-WT mice presented increased time of immobility compared with vehicle non-stressed-WT mice. In contrast, no differences were found between AM630 stressed-WT mice and vehicle non-stressed-WT mice. In addition, the administration of AM630 failed to produce any alteration in non-stressed-WT mice (Two-way ANOVA followed by Student Newman Keul's stress $F_{(1,30)}$= 1.562, p= 0.222; drug $F_{(1,30)}$= 1.879, p= 0.182; stress x drug $F_{(1,30)}$= 4.358, p= 0.046) (n=9-11/group) (Figure 4 B3).

On week 4, vehicle stressed-WT mice presented significant reduction of sucrose intake compared to vehicle non-stressed-WT mice. In contrast, stressed mice treated with AM630 did not show differences in the sucrose intake compared with non-stressed-WT mice. On the other hand, the administration of AM630 failed to produce any alteration in non-stressed-WT mice (Two way ANOVA followed by Student Newman Keul's stress $F_{(1,21)}$= 42.042, $p<0.001$; drug $F_{(1,21)}$= 13.395, p=0.002; drug x genotype $F_{(1,21)}$= 31.908, $p<0.001$) (n= 9-11/group) (Figure 4 B4). These results suggest that 4-weeks AM630 treatment was appropriated to achieve a significant improvement in depressive-like behaviors.

3.5 Analysis of BDNF expression in CB2xP and WT mice exposed to the CMS procedure and treated with AM630

BDNF gene expression was significantly reduced in vehicle stressed-WT compared with vehicle non-stressed-WT mice. In contrast, treatment with AM630 significantly blocked the reduction of BDNF mRNA levels induced by CMS procedure compared to vehicle stressed group. Interestingly, administration of AM630 by itself failed to produce any alteration in BDNF gene expression (two way ANOVA followed by Student Newman Keul's, stress $F_{(1,26)}$= 4.931, p=0.037; drug $F_{(1,26)}$= 40.393, p<0.001, stress x drug $F_{(1,26)}$= 17.310, p<0.001) (n= 10-12/group) (Figure 5 A1).

In agreement with these results, BDNF protein expression was reduced in DG of vehicle stressed and vehicle non-stressed-WT mice. In addition, the administration of AM630 completely blocked the reduction of BDNF protein levels induced by CMS. AM630 administration alone failed to produce any alteration in BDNF protein (two way ANOVA followed by Student Newman Keul's, stress $F_{(1,35)}$= 1.913, p= 0.176; drug $F_{(1,35)}$= 13.148, p<0.001, stress x drug $F_{(1,35)}$= 17.656, p<0.001) (n= 5-6/group) (Figure 5 A2 and A3).

4. DISCUSSION

The results of the present study provide unequivocal information involving the CB2r in the regulation of depressive-like behaviors. This assumption is supported by several facts: 1) The presence of basal CB2r gene expression in areas related to stress and depression in WT mice, 2) The overexpression of CB2r produced a behavioral endophenotype resistant to acute and chronic anxiogenic-like stimuli, 3) CB2xP mice presented higher BDNF gene expression in the hippocampus and 4) Treatment with AM630 blocked or significantly reduced signs of depressive-like behavior and BDNF loss in the hippocampus after chronic exposure to anxiogenic-like stimuli in WT mice.

This study describes for the first time the distribution of CB2r gene expression in different brain nuclei of WT mice under normal conditions (CPu, Acc, Cg, Amy, Hipp, VMN, ARC, SN, DR and MnR). The identification of CB2r gene expression in these brain regions predicts the role of these receptors in a wide variety of physiological functions. For instance, functional expression of these receptors in areas related to stress, anxiety and depression, such as Amy, Hipp, DR and MnR, further supports its potential role in the regulation of anxiety and depressive-like disorders.

To evaluate the implication of CB2r in the regulation of depressive-like behavior CB2xP mice were used in a variety of experimental paradigms. Previous studies evaluating the role of this receptor in the regulation of neuropathic pain have partially described these transgenic CB2xP mice (32-33). In the present study, CB2r gene expression was enhanced in the different brain nuclei analyzed (CPu, Acc, Cg, Amy, Hipp, VMN, ARC, SN, DR and MnR) of CB2xP mice. Furthermore, a significant increase of CB2r protein in the DG, CA3 and CA2 of hippocampus was also found in CB2xP mice.

The results of the analyses in the TST and NSFT revealed that CB2xP mice display a behavioral endophenotype resistant to acute anxiogenic-like stimulus. This response was characterized by decreased time of immobility, lower time of latency and higher amount of food consumption of CB2xP mice in TST and NSFT, respectively.

The effects of chronic anxiogenic-stimuli were examined exposing CB2xP mice to CMS. This animal model of depression involves the presentation of repeated unpredictable mild stressors for several weeks (34-35). Following such exposure, animals exhibited a persistent reduction in the responsiveness to pleasurable stimuli such as a palatable sucrose solution (36-38). CMS produced depressive-like behavioral alterations in WT mice (reduced time of immobility in TST and sucrose intake). In contrast, exposure to CMS failed to produce any alteration in CB2xP mice. Interestingly,

CB2xP mice (under basal conditions) presented higher sucrose intake levels than WT mice. Although the precise mechanisms underlying these basal differences remain to be elucidated, it is tempting to speculate that CB2xP mice may present neuroendocrine and/or peripheral alterations that may be disrupting the homeostatic regulation of glucose, therefore modifying the appetite of these mice.

Exposure to CMS decreased neurogenesis and produced alterations of the remodeling dendrites process in hippocampus (39-42). In this respect, BDNF plays an important role in adult neurogenesis modulating plasticity and survival of adult neurons and glia cells (43). Converging evidences revealed a reduction of hippocampal BDNF in rodents exposed to CMS (28;44-45). Moreover, clinical studies showed a reduction of hippocampal neurogenesis in patients with mood disorders (46-47). In agreement with these findings, the results of this study revealed that CMS reduced hippocampal BDNF gene and protein expressions in stressed-WT mice. In contrast, CMS failed to produce any alteration in BDNF gene and protein expressions in CB2xP mice. Indeed, CB2xP mice presented higher BDNF gene expression in the hippocampus than WT mice. The nature of the precise molecular alterations produced in CB2xP mice is still unknown. However, these findings point the CB2r as an important target involved in the “normalization” of the reduced BDNF expression in the hippocampus of mice exposed to CMS.

If overexpression of CB2r produces molecular adaptations that are associated with an endophenotype resistant to acute and chronic anxiogenic-like stimuli, it is possible to hypothesize that pharmacological manipulation with a cannabinoid CB2 antagonist (which treatment would probably increase the number of CB2r (48-49)) would produce similar effects in WT mice. In order to explore this hypothesis, the effects of AM630 were assessed in different behavioral paradigms.

The administration of AM630 (1 and 3 mg/kg) reduced the time of immobility (FST) in WT mice. These doses were without effects on the motor activity of WT mice in the open-field test (data not shown). Interestingly, the administration of AM630 did not alter the time of immobility in CB2xP mice at any of the tested doses. It is possible to propose that the lack of effects of AM630 in CB2xP mice was due to the increased expression of CB2r in these mice.

The effects of chronic (4 weeks) administration with AM630 reversed the reduction of immobility time in the TST and the decrease of sucrose intake, both induced by CMS. Furthermore, this treatment significantly blocked the reduction of BDNF gene and protein expressions in the hippocampus observed in CMS. Interestingly, the administration of AM630 increased BDNF gene

expression in the stressed mice although this alteration was not occurring at the level of protein. The reasons that may explain why the blockade of CB2r increased BDNF gene expression only in chronically stressed mice are still unknown. It can be hypothesized that AM630 act in a different manner depending on the level of activity of BDNF and CB2r. Under basal conditions (non-stress) the blockade of the receptor is not affecting the homeostatic regulation between CB2r and BDNF. In contrast, CMS would presumably increases the release of endogenous cannabinoid ligands, which in turn, could tend to down regulate CB2r and decreased BDNF expression. These changes may result in hypersensitization of the CB2r, and through an unknown mechanism, could be responsible of the pronounced increase of BDNF after treatment with AM630. Further studies are needed to identify the precise neurochemical mechanisms by which the CB2r regulates BDNF gene expression during stress.

In summary, the results presented here show that CB2r play a pivotal role in the neurobiology of depressive related disorders. Overexpression of CB2r resulted in a behavioral endophenotype resistant to anxiogenic-like stimulus and modified different targets involved in the neuroplasticity of depressive disorders, such as BDNF. In addition, the acute and chronic treatment with AM630 resulted in antidepressant-like effects and reversed the reduction of BDNF induced in the hippocampus of mice exposed to CMS. Taken together, these findings strongly support the role of CB2r in the regulation of depressive disorders and point out this receptor as a new potential key target in the treatment of different mood-related disorders.

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REFERENCES

1. Griebel G, Stemmelin J, Scatton B (2005): Effects of the cannabinoid CB1 receptor antagonist rimonabant in models of emotional reactivity in rodents. *Biol Psychiatry* 57:261-267.
2. Witkin JM, Tzavara ET, Davis RJ, Li X, Nomikos GG (2005): A therapeutic role for cannabinoid CB1 receptor antagonists in major depressive disorders. *Trends Pharmacol Sci* 26:609-617.
3. Shearman LP, Rosko KM, Fleischer R, Wang J, Xu S, Tong XS, et al (2003): Antidepressant-like and anorectic effects of the cannabinoid CB1 receptor inverse agonist AM251 in mice. *Behav Pharmacol* 14:573-582.
4. Tzavara ET, Davis RJ, Perry KW, Li X, Salhoff C, Bymaster FP, et al (2003): The CB1 receptor antagonist SR141716A selectively increases monoaminergic neurotransmission in the medial prefrontal cortex: implications for therapeutic actions. *Br J Pharmacol* 138:544-553.
5. Need AB, Davis RJ, Alexander-Chacko JT, Eastwood B, Chernet E, Phebus LA, et al (2006): The relationship of in vivo central CB1 receptor occupancy to changes in cortical monoamine release and feeding elicited by CB1 receptor antagonists in rats. *Psychopharmacology (Berl)* 184:26-35.
6. Le Foll B, Gorelick DA, Goldberg SR (2009): The future of endocannabinoid-oriented clinical research after CB(1) antagonists. *Psychopharmacology (Berl)*.
7. Moreira FA, Grieb M, Lutz B (2009): Central side-effects of therapies based on CB1 cannabinoid receptor agonists and antagonists: focus on anxiety and depression. *Best practice & research* 23:133-144.
8. Adamczyk P, Golda A, McCreary AC, Filip M, Przegalinski E (2008): Activation of endocannabinoid transmission induces antidepressant-like effects in rats. *J Physiol Pharmacol* 59:217-228.
9. Bambico FR, Gobbi G (2008): The cannabinoid CB1 receptor and the endocannabinoid anandamide: possible antidepressant targets. *Expert opinion on therapeutic targets* 12:1347-1366.
10. Rodriguez-Gaztelumendi A, Rojo ML, Pazos A, Diaz A (2009): Altered CB receptor-signaling in prefrontal cortex from an animal model of depression is reversed by chronic fluoxetine. *J Neurochem* 108:1423-1433.
11. Hill MN, Carrier EJ, McLaughlin RJ, Morrish AC, Meier SE, Hillard CJ, et al (2008): Regional alterations in the endocannabinoid system in an animal model of depression: effects of concurrent antidepressant treatment. *J Neurochem* 106:2322-2336.

12. Hungund BL, Basavarajappa BS (2004): Role of endocannabinoids and cannabinoid CB1 receptors in alcohol-related behaviors. *Ann N Y Acad Sci* 1025:515-527.
13. Benito C, Nunez E, Tolon RM, Carrier EJ, Rabano A, Hillard CJ, et al (2003): Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. *J Neurosci* 23:11136-11141.
14. Yiangou Y, Facer P, Durrenberger P, Chessell IP, Naylor A, Bountra C, et al (2006): COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord. *BMC neurology* 6:12.
15. Guzman M, Sanchez C, Galve-Roperh I (2001): Control of the cell survival/death decision by cannabinoids. *J Mol Med* 78:613-625.
16. Joosten M, Valk PJ, Jorda MA, Vankan-Berkhoudt Y, Verbakel S, van den Broek M, et al (2002): Leukemic predisposition of pSca-1/Cb2 transgenic mice. *Experimental hematology* 30:142-149.
17. Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, et al (2005): Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* 310:329-332.
18. Gong JP, Onaivi ES, Ishiguro H, Liu QR, Tagliaferro PA, Brusco A, et al (2006): Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res* 1071:10-23.
19. Onaivi ES (2006): Neuropsychobiological evidence for the functional presence and expression of cannabinoid CB2 receptors in the brain. *Neuropsychobiology* 54:231-246.
20. Onaivi ES, Ishiguro H, Gong JP, Patel S, Meozzi PA, Myers L, et al (2008): Brain neuronal CB2 cannabinoid receptors in drug abuse and depression: from mice to human subjects. *PLoS ONE* 3:e1640.
21. Onaivi ES, Ishiguro H, Sejal P, Meozzi PA, Myers L, Tagliaferro P, et al (2006): Methods to study the behavioral effects and expression of CB2 cannabinoid receptor and its gene transcripts in the chronic mild stress model of depression. *Methods Mol Med* 123:291-298.
22. Pertwee R, Griffin G, Fernando S, Li X, Hill A, Makriyannis A (1995): AM630, a competitive cannabinoid receptor antagonist. *Life sciences* 56:1949-1955.
23. Hosohata Y, Quock RM, Hosohata K, Makriyannis A, Consroe P, Roeske WR, et al (1997): AM630 antagonism of cannabinoid-stimulated [35S]GTP gamma S binding in the mouse brain. *European journal of pharmacology* 321:R1-3.

24. Vaugeois JM, Passera G, Zuccaro F, Costentin J (1997): Individual differences in response to imipramine in the mouse tail suspension test. *Psychopharmacology* 134:387-391.
25. Cryan JF, Markou A, Lucki I (2002): Assessing antidepressant activity in rodents: recent developments and future needs. *Trends Pharmacol Sci* 23:238-245.
26. Willner P, Muscat R, Papp M (1992): An animal model of anhedonia. *Clinical neuropharmacology* 15 Suppl 1 Pt A:550A-551A.
27. Ducottet C, Aubert A, Belzung C (2004): Susceptibility to subchronic unpredictable stress is related to individual reactivity to threat stimuli in mice. *Behavioural brain research* 155:291-299.
28. Li S, Wang C, Wang M, Li W, Matsumoto K, Tang Y (2007): Antidepressant like effects of piperine in chronic mild stress treated mice and its possible mechanisms. *Life sciences* 80:1373-1381.
29. Paxinos G, Franklin, K.B.J. (2001): *The mouse brain in stereotaxic coordinates*. New York: Academic Press. Harcourt Science and Technology Company.
30. Palkovits M (1983): Punch sampling biopsy technique. *Methods in enzymology* 103:368-376.
31. Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif* 25:402-408.
32. Racz I, Nadal X, Alferink J, Banos JE, Rehnelt J, Martin M, et al (2008): Interferon-gamma is a critical modulator of CB(2) cannabinoid receptor signaling during neuropathic pain. *J Neurosci* 28:12136-12145.
33. Racz I, Nadal X, Alferink J, Banos JE, Rehnelt J, Martin M, et al (2008): Crucial role of CB(2) cannabinoid receptor in the regulation of central immune responses during neuropathic pain. *J Neurosci* 28:12125-12135.
34. Willner P (1997): Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology (Berl)* 134:319-329.
35. Papp M, Moryl E, Willner P (1996): Pharmacological validation of the chronic mild stress model of depression. *Eur J Pharmacol* 296:129-136.
36. D'Aquila PS, Brain P, Willner P (1994): Effects of chronic mild stress on performance in behavioural tests relevant to anxiety and depression. *Physiol Behav* 56:861-867.
37. Forbes NF, Stewart CA, Matthews K, Reid IC (1996): Chronic mild stress and sucrose consumption: validity as a model of depression. *Physiol Behav* 60:1481-1484.

38. Willner P, Muscat R, Papp M (1992): Chronic mild stress-induced anhedonia: a realistic animal model of depression. *Neurosci Biobehav Rev* 16:525-534.
39. Manji HK, Drevets WC, Charney DS (2001): The cellular neurobiology of depression. *Nat Med* 7:541-547.
40. Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM (2002): Neurobiology of depression. *Neuron* 34:13-25.
41. Nestler EJ, Gould E, Manji H, Buncan M, Duman RS, Greshenfeld HK, et al (2002): Preclinical models: status of basic research in depression. *Biol Psychiatry* 52:503-528.
42. Coyle JT, Duman RS (2003): Finding the intracellular signaling pathways affected by mood disorder treatments. *Neuron* 38:157-160.
43. Huang EJ, Reichardt LF (2001): Neurotrophins: roles in neuronal development and function. *Annual review of neuroscience* 24:677-736.
44. Li S, Wang C, Wang W, Dong H, Hou P, Tang Y (2008): Chronic mild stress impairs cognition in mice: from brain homeostasis to behavior. *Life sciences* 82:934-942.
45. Toth E, Gersner R, Wilf-Yarkoni A, Raizel H, Dar DE, Richter-Levin G, et al (2008): Age-dependent effects of chronic stress on brain plasticity and depressive behavior. *J Neurochem* 107:522-532.
46. Sheline YI, Wang PW, Gado MH, Csernansky JG, Vannier MW (1996): Hippocampal atrophy in recurrent major depression. *Proc Natl Acad Sci U S A* 93:3908-3913.
47. Sheline YI (2000): 3D MRI studies of neuroanatomic changes in unipolar major depression: the role of stress and medical comorbidity. *Biol Psychiatry* 48:791-800.
48. Neisewander JL, Nonneman AJ, McDougall SA, Bardo MT (1989): Up-regulation of opiate receptors following chronic naloxone treatment in aged rats. *Neurobiology of aging* 10:55-58.
49. Patel M, Gomes B, Patel C, Yoburn BC (2002): Antagonist-induced micro-opioid receptor up-regulation decreases G-protein receptor kinase-2 and dynamin-2 abundance in mouse spinal cord. *Eur J Pharmacol* 446:37-42.

FIGURE LEGENDS

Figure 1. CB2 receptor gene expression in CB2xP and WT mice. A) Relative CB2 receptor gene expression in different brain nuclei of CB2xP and WT mice by Rt-PCR (CPu: caudate-putamen nucleus, Acc: nucleus accumbens, Cg: Cingulated cortex, Amy: Amygdala, Hipp: hippocampus, VMN: ventromedial nucleus of hypothalamus, ARC: arcuate nucleus of hypothalamus, Sn: substantia nigra, VTA: ventral tegmental area; DR: dorsal raphe, MnR: medial raphe). Relative expression was calculated according to the $\Delta\Delta$Ct method. Columns represent the means and vertical lines the $\pm$ SEM of relative CB2 gene expression, * Values from different brain WT mice regions that are significantly different from CPu WT mice, $^{\&}$ Values from CB2xP mice (black columns) in each brain region that are significantly different ($p<0.05$, Student t-test) compared to its corresponding region of WT mice (white columns) (n= 6-7 per group). B) CB2 receptor immunostaining in different fields of hippocampus in CB2xP and WT mice. DG: Dentate gyrus. * Values from CB2xP mice (black columns) that are significantly different ($p<0.05$, Student t-test) from WT mice (white columns) (n= 4 per group). C) Representative autoradiograms for CB2 protein in hippocampus of WT and CB2xP mice; bar represent 1 mm.

Figure 2. Assessment of depressive-like behaviors in CB2xP and WT mice in tail suspension (A), novelty suppressed feeding (B) and chronic unpredictable mild stress paradigms (C). Columns represent the means and vertical lines $\pm$ SEM of A (time of immobility (s)) and B (time of latency (s) and (consumption of food pellets (g)) in 11-14 mice; * Values from CB2xP mice (black columns) that are significantly different from WT mice (white columns) ($p<0.05$, Student t-test). During the CMS procedure, depressive-like behaviors were evaluated by using tail suspension (5 week) (C2) and sucrose intake tests (7 week) (C3) (n= 12) (See methods). Columns represent the means and vertical lines $\pm$ SEM of time of latency (s) (C2) and sucrose intake (mg sacarose/g body weight) (C3) (n= 12-16 mice per group). $^{\&}$ Values from stressed-WT mice that are significantly different from non-stressed WT mice ($p<0.05$, Student t-test). Bw: Body weight.

Figure 3. BDNF gene and protein expressions in CB2xP and WT mice exposed to CMS. A1) Relative BDNF mRNA levels in hippocampus of CB2xP and WT mice exposed to CMS. Columns represent the means and vertical lines the ± SEM of relative BDNF mRNA gene expression, * Values from non-stressed-WT, stressed-CB2xP and non-stressed-CB2xP mice that are significantly different from non-stressed-WT group (two way ANOVA, followed by Student Newman Keul's, $p<0.05$) (n=5-7 per group). A2) Quantification of BDNF immunostaning in hippocampus of CB2xP and WT mice exposed to CMS. Columns represent the means and vertical lines the ± SEM of relative BDNF protein (arbritrary units). * Values from stressed-WT mice that are significantly different from non-stressed-WT mice (two way ANOVA, followed by Student Newman Keul's, $p<0.05$) (n= 5-6 per group). A3) Representative autoradiograms for BDNF protein in hippocampus of CB2xP and WT mice exposed to CMS. Bar represent 1 mm.

Figure 4. Acute and chronic effects of cannabinoid CB2 antagonist AM630 treatment. A) Dose-response effects of AM630 on forced swimming test in WT and CB2xP mice. Columns represent the means and vertical lines the ± SEM of time of immobility (s). * Values from AM630-WT group (1 and 3 mg/kg, i.p.) that are significantly different from vehicle-WT group (one way ANOVA followed by Student Newman Keul's, $p<0.05$) (n=6-8). B) Chronic effects of AM630 treatment in WT mice exposed to CMS paradigm. Evaluation of depressive-like behaviors on weeks 3 and 4 of the CMS paradigm by using tail suspension (B1) and sucrose intake (B2) tests, respectively (n= 24). Evaluation of depressive-like behaviors on weeks 3 and 4 after chronic AM630 treatment by using tail suspension (B3) and sucrose intake (B4) tests, respectively (n= 9-11) (see methods). Columns represent the means and vertical lines the ± SEM of time of immobility (s) (B1, B3) and sucrose intake (mg sucrose/ g body weight) (B2, B4). * Values from stress-WT group that are significantly different from non-stressed-WT group (two way ANOVA followed Studen't Newman Keul's, $p<0.05$), $^{\#}$ Values from vehicle stressed-WT group that are significantly different from from vehicle non-stressed-WT group (two way ANOVA followed Studen't Newman Keul's, $p<0.05$). Bw: Body weight.

Figure 5. BDNF gene and protein expressions in chronic AM630 treated WT mice exposed to CMS. A1) Relative BDNF mRNA levels in hippocampus of AM630 and vehicle WT mice exposed to CMS. Columns represent the means and vertical lines the ± SEM of relative BDNF mRNA gene expression, * Values from vehicle stressed-WT and AM630 stressed-WT groups that are significantly different from vehicle non-stressed-WT group (two way ANOVA, followed by Student Newman Keul's, $p<0.05$) (n= 10-11 per group). A2) BDNF immunostaining in the hippocampus of AM630 and vehicle WT mice exposed to CMS. Columns represent the means and vertical lines the ± SEM of relative BDNF protein (arbritrary units). * Values from vehicle stressed-WT group that are significantly different from vehicle non-stressed-WT group (two way ANOVA, followed by Student Newman Keul's, $p<0.05$) (n= 5-6 per group). A3) Representative autoradiograms for BDNF protein in hippocampus WT stressed and non-stressed AM630 and vehicle mice. Bar represent 1 mm.

Table 1. Chronic unpredictable mild stress procedure

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 1	10-12h Strong noise 14-17h Stroboscopic illumination 18-19h Restraint stress	10-11h Restraint stress 14-16h Strong noise 19-8.30h Food deprivation	10-13.30h Stroboscopic illumination 16-17h Restraint stress 19-8.30h Tilted cage	10-11h Restraint stress 14-16h Strong noise 19-8.30h Wet cage	10-13h Stroboscopic illumination 16-17h Restraint stress 19-19h Tilted cage	19-8.30h Food deprivation	8.30-8.30h Inversion light/dark cycle
Week 2	10-11h Restraint stress 14-17h Stroboscopic illumination 19-8.30h Wet cage	10-13.30h Stroboscopic illumination 16-17h Restraint stress 19-8.30h Tilted cage	10-12h Strong noise 14-17h Stroboscopic illumination 18-19h Restraint stress	8.30-13h Behavioural test= Light-Dark box 15-17h Strong noise 18-19h Restraint stress	10-13.30h Stroboscopic illumination 19-8.30h Food deprivation	8.30-19h Inversion light/dark cycle	19-8.30h Wet cage
Week 3	10-12h Strong noise 14.30-15.30h Restraint stress 19-8.30h Tilted cage	10-11h Restraint stress 14-17h Stroboscopic illumination 17.30-19.30h Strong noise	10-12h Strong noise 14-15h Restraint stress 19-8.30h Food deprivation	8.30-13h Behavioural test= Elevated Plus Maze 15-18h Stroboscopic illumination 19-8.30h Wet cage	10-13.30h Stroboscopic illumination 15-16h Restraint stress 17-19h Strong noise	8.30-19h Inversion light/dark cycle	19-8.30h Food deprivation
Week 4	8.30-13h Behavioural test= Tail Suspension Test 14-16h Strong noise 18-19h Restraint stress	10-13.30h Stroboscopic illumination 16-17h Restraint stress 19-8.30h Tilted cage	10-11h Restraint stress 14-17h Stroboscopic illumination 19-8.30h Wet cage	10-12h Strong noise 15-18h Stroboscopic illumination	10-12h Strong noise 16-17h Restraint stress 19-19h Tilted cage	19-8.30h Food deprivation	8.30-8.30h Inversion light/dark cycle
Week 5	10-11h Restraint stress 14-16h Strong noise 19-8.30h Wet cage	11-14.30h Stroboscopic illumination 16-18h Strong noise	10-11h Restraint stress 14-16h Strong noise 19-8.30h Tilted cage	11-14.30h Stroboscopic illumination 16-17h Restraint stress 19-8.30h Wet cage	10-12h Strong noise 14-17h Stroboscopic illumination 18-19h Restraint stress	8.30-18.00h Tilted cage 19-8.30h Food deprivation	8.30-8.30h Inversion light/dark cycle
Week 6	10-12h Strong noise 16-17h Restraint stress 19-8.30h Wet cage	10-13.30h Stroboscopic illumination 16-17h Restraint stress 19-8.30h Tilted cage	10-12h Strong noise 14-15h Restraint stress 19-8.30h Food deprivation	10-11h Restraint stress 14-16h Strong noise 19-13h Food and water deprivation	13-14h Behavioural test= Sucrose Intake		

Table 2. CB2 gene expression (relative to CPu) in WT mice

REGIONS	Student t-test
Acc	t= -3.099, p<0.011, 10 df * (137%)
Cg	t= -0.688, p<0.505, 11 df
Amy	t= -2.410, p<0.037, 10 df * (107%)
Hipp	t= -0.226, p<0.825, 13 df
VMN	t=-2.849, p<0.017, 10 df * (90%)
ARC	t= -1.847, p<0.092, 11 df
Sn	t= -3.125, p<0.010, 11 df * (224%)
VTA	Non detected
DR	t= -0.568, p=0.582, 10 df
MnR	t= -2.190, p=0.050, 11 df * (84%)

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