Non-psychoactive CB_2 cannabinoid agonists stimulate neural progenitor proliferation

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ABSTRACT **Cannabinoids, the active components of marijuana and their endogenous counterparts, act on the brain and many other organs through the widely expressed CB_1 cannabinoid receptor. In contrast, the CB_2 cannabinoid receptor is abundant in the immune system and shows a restricted expression pattern in brain cells. CB_2-selective agonists are, therefore, very attractive therapeutic agents as they do not cause CB_1-mediated psychoactive effects. CB_2 receptor expression in brain has been partially examined in differentiated cells, while its presence and function in neural progenitor cells remain unknown. Here we show that the CB_2 receptor is expressed, both *in vitro* and *in vivo*, in neural progenitors from late embryonic stages to adult brain. Selective pharmacological activation of the CB_2 receptor *in vitro* promotes neural progenitor cell proliferation and neurosphere generation, an action that is impaired in CB_2-deficient cells. Accordingly, *in vivo* experiments evidence that hippocampal progenitor proliferation is increased by administration of the CB_2-selective agonist HU-308. Moreover, impaired progenitor proliferation was observed in CB_2-deficient mice both in normal conditions and on kainate-induced excitotoxicity. These findings provide a novel physiological role for the CB_2 cannabinoid receptor and open a novel therapeutic avenue for manipulating neural progenitor cell fate.—Palazuelos, J., Aguado, T., Egia, A., Mechoulam, R., Guzmán, M., Galve-Roperh, I. Non-psychoactive CB_2 cannabinoid agonists stimulate neural progenitor proliferation. *FASEB J.* 20, E1773–E1779 (2006)**

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The hemp plant *Cannabis sativa* produces ~70 unique compounds known as cannabinoids, of which Δ^9-tetrahydrocannabinol (THC) is the most important owing to its high potency and abundance in cannabis. THC exerts a wide variety of biological effects by mimicking endogenous substances—the endocannabinoids anandamide (1) and 2-arachidonoylglycerol (2)—that bind to and activate specific cannabinoid receptors. So far, two cannabinoid-specific G protein-coupled receptors have been cloned and characterized from mammalian tissues: CB_1 and CB_2 (3, 4). It is well established that the central and most of the peripheral effects of cannabinoids rely on CB_1 receptor activation (4). This receptor is highly abundant in the central nervous system (CNS) and is expressed by the major types of brain cells [neurons (5), astrocytes (6), oligodendrocytes (7), and microglia (8)]. In particular, CB_1 receptors present in central neurons that control processes such as motor activity, memory and cognition, pain, emotion, sensorial perception, and endocrine functions are targets for the neuromodulatory action of endocannabinoids, as well as for the psychoactive effects of marijuana-derived cannabinoids (4). Functionally active CB_1 receptors are also expressed in peripheral nerve terminals, and various extraneural sites such as testis, eye, vascular endothelium, and spleen (3, 4).

The CB_2 receptor displays a more limited pattern of expression than the CB_1 receptor, which is found almost exclusively in cells (*e.g.*, B- and T-lymphocytes, macrophages) and tissues (*e.g.*, spleen, tonsils, lymph nodes) of the immune system (9). Within the brain, the CB_2 receptor is expressed only in perivascular microglial cells (10), vascular endothelial cells (11), and certain neuron subpopulations (12–14). This restricted expression pattern in the brain, however, makes the CB_2 receptor an interesting therapeutic target since the unwanted psychotropic effects of cannabinoids, which severely limit their medical use, are mediated largely or entirely by neuronal CB_1 receptors (4). While CB_2 receptor expression in brain has been examined to date only in differentiated cells, the presence and function of this receptor in neural progenitor cells remain unknown. Here we show that CB_2 receptors are expressed in neural progenitors and that its selective activation stimulates cell proliferation. This finding provides a new conceptual view in the understanding of how the endocannabinoid system signals in brain and how neural progenitor proliferation is controlled, and it points to the potential pharmacological modulation

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of neural progenitor cell fate by psychoactivity-devoid CB_2-selective ligands.

MATERIALS AND METHODS

Materials

The following materials were kindly donated: CB_2 receptor knockout mice by Nancy Buckley (National Institute of Health, Bethesda, MD, USA), HU-308 by Pharmos (Rehovot, Israel), JWH-133 by John W. Huffman (Clemson University, Clemson, NC, USA), SR144528 by Sanofi-Aventis (Montpellier, France), and anti-mouse phosphorylated-S55 vimentin monoclonal 4A4 antibody (Ab) by Verónica Cerdeño (University of California San Francisco, CA, USA). Anti-CB_2 receptor polyclonal antibody (pAb) was from Affinity Bioreagents (Golden, CO, USA). Mouse monoclonal antinestin Ab was from Chemicon (Temecula, CA, USA), and mouse monoclonal anti-NeuN, anti-GFAP, anti-α-tubulin antibodies were from Sigma (St. Louis, MO, USA). Rat monoclonal antibromodeoxyuridine Ab was from Abcam (Cambridge, UK) and monoclonal anti-RC2 Ab was from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). Sheep polyclonal antiphosphoY180-extracellular signal-regulated kinase (ERK)1/2 was from Upstate Biotechnology (Lake Placid, NY, USA) and rabbit polyclonal anti-Akt, phosphoS473-Akt and anti-ERK1/2 were from Cell Signaling Technology (Beverly, MA, USA). PD98059 and LY294,002 were from Alexis Biochemicals (San Diego, CA, USA).

Neurosphere and neural progenitor cell culture

Multipotent self-renewing progenitors were obtained from the dissected cortices of embryonic mice at the indicated developmental stages, subventricular zone in adult brain, and grown in chemically defined medium constituted by Dulbecco's modified Eagle's and F12 media supplemented with N2 (Invitrogen, Carlsbad, CA, USA), 0.6% glucose (Glc), nonessential amino acids, 50 mM HEPES, 2 μg/ml heparin, 20 ng/ml epidermal growth factor (EGF), and 20 ng/ml basic fibroblast growth factor (bFGF) (15). Clonal neurospheres were cultured at 1000 cells/ml, dissociated, and experiments were performed with early (up to 10) passage neurospheres. Neurosphere generation experiments were performed in 96-well dishes with 100 μl of medium, and the number of neurospheres was quantified. Embryonic neural progenitors from wild-type (WT) and CB_2-deficient mice were cultured (10,000 cells/ml) in the continuous presence of cannabinoids for the indicated number of passages (1 passage every 4 d). Adult neural progenitors were obtained from hippocampi of 4-month-old adult mice and cultured as described above. Neural progenitor cell differentiation was performed as described (15). Stock solutions of cellular effectors were prepared in dimethyl sulfoxide. No significant influence of dimethyl sulfoxide on any of the parameters determined was observed at the final concentration used (0.1% v/v). Control incubations included the corresponding vehicle content.

Cell proliferation assays

Neural progenitor proliferation was determined by quantifying bromodeoxyuridine (BrdU)-positive cells 16 h after incubation with 10 μg/ml BrdU, followed by immunostaining (16).

Western blot

Cleared cell extracts were subjected to SDS-PAGE, transferred to PVDF membranes, and following Ab incubations developed with enhanced chemiluminiscence detection kit (16). Loading controls were performed with an anti-α-tubulin Ab.

RT-polymerase chain reaction (RT-PCR)

RNA was obtained with the RNeasy Protect kit (Qiagen, Valencia, CA) using the RNase-free DNase kit. cDNA was subsequently obtained using the Superscript First-Strand cDNA synthesis kit (Roche, Welwyn Garden City, UK), and amplification of cDNA was performed with the following primers: mouse CB_2, sense GGATGCCGGGAGACAGAAGTGA and antisense CCCATGAGCGGCAGGTAAGAAAT (506 bp product); human CB_2, sense, CAACCCAAAGCCTTCTAGACAAG and antisense GTGGATAGCGCAGGCAGAGGT (464 bp product). Mouse and human CB_2 polymerase chain reaction (PCR) reactions were performed using the following conditions: 1 min at 95°C and 35 cycles (30s at 95°C, 30s at 58°C, and 1 min at 72°C). Finally, after a final extension step at 72°C for 5 min, PCR products were separated on 1.5% agarose gels.

Animals and drug treatment

Adult CB_2 receptor knockout mice (8 weeks old) and their respective WT littermates were injected i.p. with 50 mg/kg BrdU daily for 3 d and perfused 1 d later. HU-308 (15 mg/kg) was administered i.p. for 5 d either alone or in combination with 1 mg/kg SR144528 (injected 30 min before HU-308). Control animals received the corresponding vehicle injection (100 μl PBS supplemented with 0.5 mg defatted BSA and 4% dimethyl sulfoxide). BrdU was administered daily during the pharmacological administration period. In the case of experiments on kainate-induced excitotoxicity, animals were injected with 15 mg/kg kainate or vehicle. E17.5 mouse embryos from mothers injected twice with 100 mg/kg BrdU (30-min interval between injections) were obtained 1 h after the first injection. Animal procedures were performed according to the European Union guidelines (86/609/EU) for the use of laboratory animals.

Immunostaining and confocal microscopy

Mice were perfused and immunostaining was performed in 30 μm brain coronal free-floating sections (15, 17). Sections were incubated with polyclonal anti-CB_2 Ab together with anti-nestin, anti-Neu, or anti-GFAP antibodies followed by secondary staining for rabbit and mouse IgGs with highly cross-adsorbed AlexaFluor 594 and AlexaFluor 488 secondary antibodies (Molecular Probes, Eugene, OR, USA), respectively. Neural progenitor proliferation was determined with anti-bromodeoxyuridine Ab and secondary antirat IgG-AlexaFluor 594 in sections counterstained with TOTO-3 iodide. Preparations were examined using Leica TCS-SP2 software Leica (Wetzlar, Germany) and SP2 microscope with 2 passes with a Kalman filter and a 1024 × 1024 collection box. $BrdU^+$ cells were counted in the subgranular zone and granule cell layer of the dentate gyrus. A 1-in-6 series of adult hippocampal mouse sections located between 1.3 and 2.1 mm posterior to bregma were used. The number of cells was normalized to the area of the dentate gyrus of each 30-μm section followed by the determination of the total positive cell number per animal. Frozen mouse embryo sections were incubated with anti-bromodeoxyuridine Ab together with Yoyo-1 iodide, and positive cells were determined in 7 sections per animal. The specificity of CB_2 receptor immunoreactivity was corrobo-

rated using $CB_2^{-/-}$ mouse sections, in which no immunoreactivity was observed, and allowed to adjust optimal confocal microscope settings.

Statistical analysis

Results shown represent the means ± SD of the number of experiments indicated in every case. Statistical analysis was performed by ANOVA. A *post hoc* analysis was made by the Student-Neuman-Keuls test. *In vivo* data were analyzed by an unpaired Student's *t* test.

RESULTS

Neural progenitors express CB_2 receptors *in vitro* and *in vivo*

To determine whether neural progenitor cells express CB_2 receptors, we generated clonally expanded neurospheres derived from embryonic and adult brain. Reverse transcription-polymerase chain reaction (**Fig. 1*A***) and Western blot (Fig. 1*B*) analyses revealed that neural progenitors express CB_2 receptors and that their presence remains evident as well in adult-derived cells. These findings were extrapolated to human neural progenitors (Fig. 1*C*), as CB_2 is also present in the hNSC1 cell line (18). We next labeled neural progenitors with antibodies directed against the CB_2 receptor and nestin, a widely used marker of multipotent neuroepithelial cells. As inferred from the colocalization images, we confirmed that neural progenitor cells, including those actively dividing (as identified by BrdU incorporation), express CB_2 receptors (Fig. 1*D*, upper panels). Importantly, radial progenitor cells, the postulated continuum lineage from embryonic toward adult neural progenitors (19), were also positive for CB_2 receptors. Thus, cells expressing the radial glial marker RC2, as well as dividing radial cells identified by an Ab against phosphorylated vimentin, were double-labeled with the anti-CB_2 Ab (Fig. 1*D*, middle panels). In line with these observations, CB_2 receptor expression persisted in adult neural progenitor cells (Fig. 1*D*, lower panels). As CB_2 receptor expression is known to be restricted in neural cells, we next sought to investigate its potential regulation regarding neural differentiation. Thus, neural progenitors were differentiated and CB_2 expression was analyzed in parallel with β-tubulin-III and GFAP, markers of neuronal and astroglial cells, respectively. CB_2 receptor expression was abrogated in differentiated cells with the concomitant appearance of the neuronal and astroglial markers (Fig. 1*E*).

Next, we determined by confocal microscopy whether CB_2 receptors are expressed *in vivo* in progenitor cells resident in the subgranular zone of the dentate gyrus of the hippocampus, one of the most prominent neurogenic areas throughout life span, including adulthood (19, 20). As shown in **Fig. 2**, CB_2 receptor expression was found only in nestin-positive cells, while we could not find its presence in differentiated hippocampal neurons (NeuN-positive cells) and astrocytes (GFAP-positive cells). Altogether, these re-

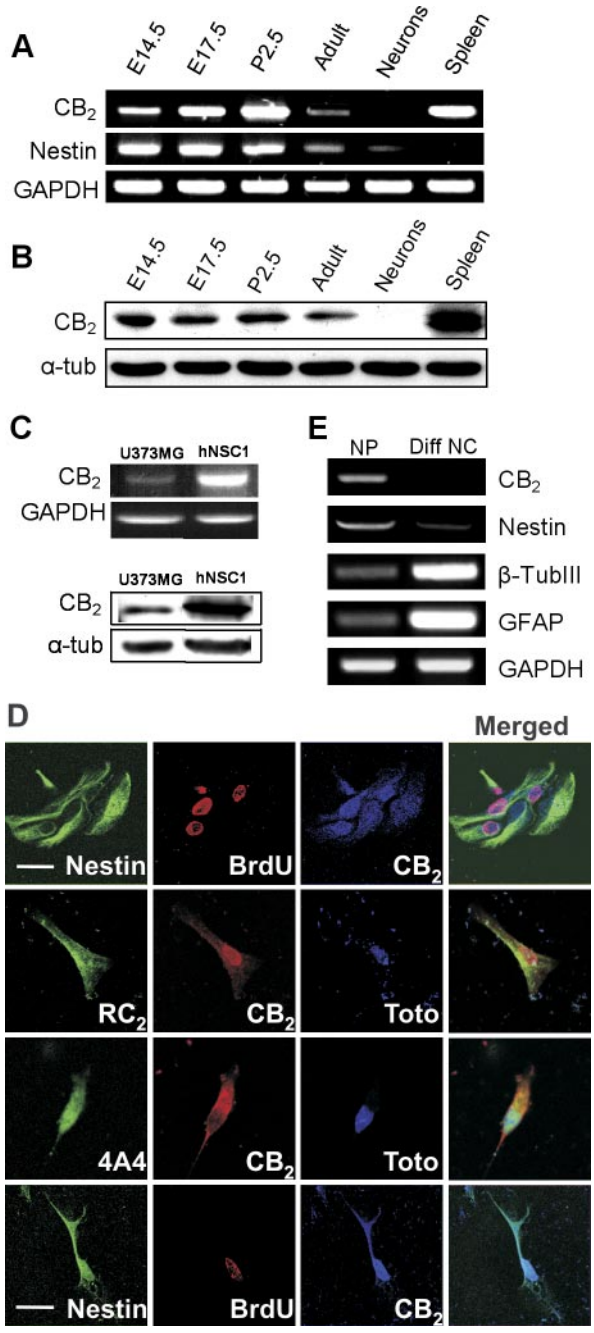

Figure 1. Neural progenitors express CB_2 receptors *in vitro*. *A)* Expression of the CB_2 receptor and nestin in embryonic (E), postnatal (P), and adult neural progenitors as determined by RT-PCR. Differentiated cortical neurons as well as spleen were used as negative and positive controls, respectively. *B)* Expression of the CB_2 receptor as above determined by Western blot. *C)* Expression of the CB_2 receptor in the human hNSC.1 stem cell line and the human U373MG astrocytoma cell line (control) as determined by RT-PCR (upper panel) and Western blot (lower panel). *D)* Adherent embryonic (upper panels) and adult (lower panels) neural progenitor cultures were immunostained with antinestin (green), BrdU (red), and CB_2 receptor (blue) antibodies. Postnatal radial glial progenitors (middle panels) were labeled against RC2 or phosphorylated-vimentin (green) and the CB_2 receptor (red). Colocalization is shown in the merged images. Scale bars 20 μm. *E)* Analysis of CB_2 receptor expression in undifferentiated neural progenitors (NP) and their differentiated neural cell progeny (Diff NC) evaluated by the presence of nestin, β-tubulin III and GFAP transcripts.

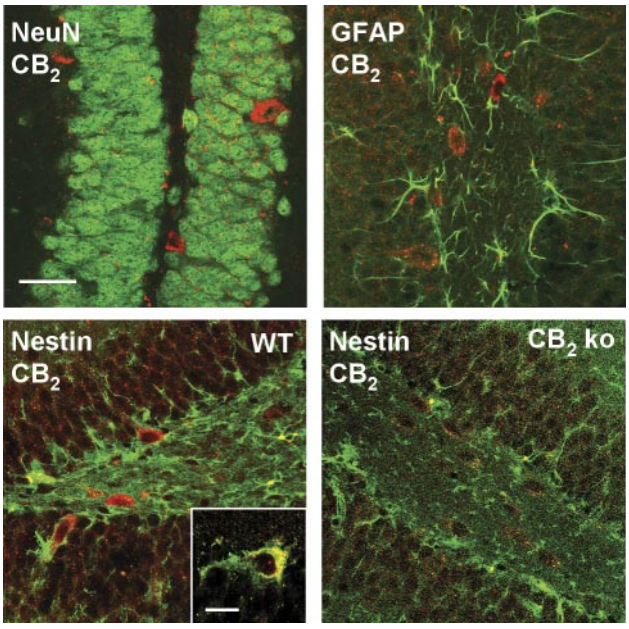

Figure 2. Neural progenitors express CB_2 receptors *in vivo*. Expression of the CB_2 receptor (red) in neural progenitors (nestin-positive cells; green) but not in mature neurons (NeuN-positive cells; green) and astrocytes (GFAP-positive cells; green) as assessed by confocal microscopy in adult hippocampal sections. Inset shows a high magnification image of a representative double nestin-CB_2 positive cell. Sections from $CB_2^{-/-}$ deficient were employed as specificity controls. Cells were counterstained with TOTO-3 iodide (blue). Scale bars: 40 and 10 μm.

sults show that CB_2 cannabinoid receptors are expressed in neural progenitor cells both during development and in adulthood and become down-regulated with neural cell differentiation.

CB_2 receptors control neural progenitor cell proliferation *in vitro*

To determine whether CB_2 receptors control neural progenitor cell function, we first generated neurospheres from CB_2-deficient mice (21) and their WT littermates. Genetic ablation of the CB_2 receptor impaired primary neurosphere generation (**Fig. 3*A***, inset). Moreover, neural progenitor self-renewal, as determined by neurosphere generation for several consecutive passages, was reduced in CB_2-deficient cells (Fig. 3*A*). The observed impairment of neural progenitor function in $CB_2^{-/-}$ cell cultures prompted us to analyze the prominin (cluster of differentiation-133)-positive subpopulation, as these cells are considered to constitute the stem cell fraction responsible for neurosphere formation activity (22). Of interest, $CB_2^{-/-}$ neurospheres, when compared to WT cultures by flow cytometry analysis, showed a reduction in their cluster of differentiation (CD)-133^+ subpopulation (cluster of differentiation-133^+ cells: 5.8±2.0% *vs.* 7.4±1.5%, respectively).

The functional relevance of the CB_2 receptor was investigated further by incubating neurospheres with

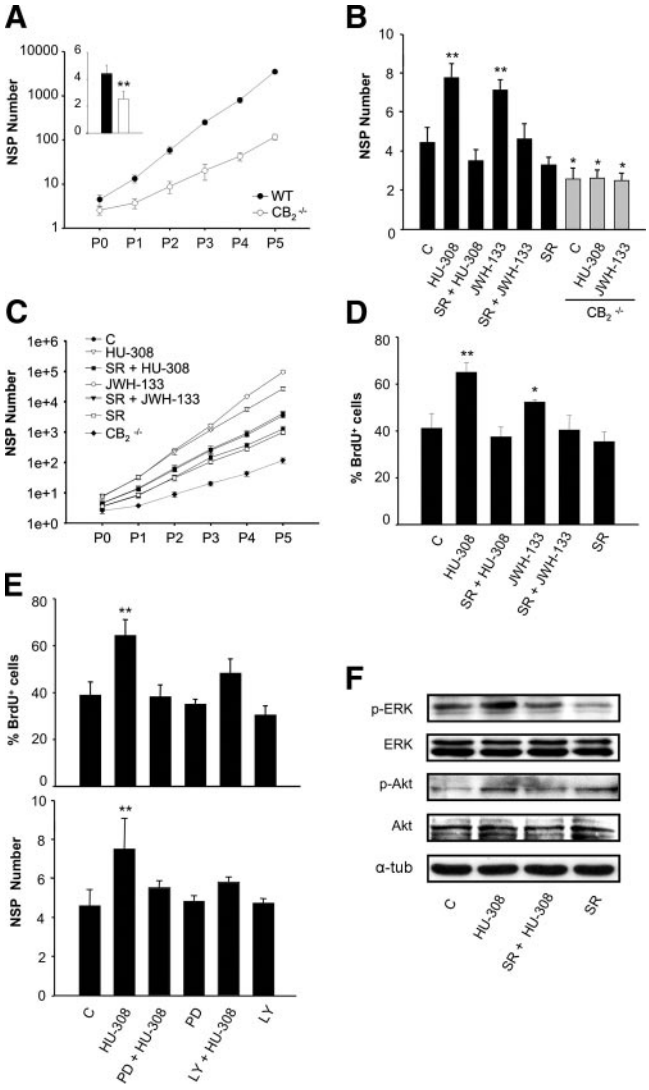

Figure 3. CB_2 receptors control neurosphere generation and neural progenitor cell proliferation *in vitro*. *A)* Self-renewal of E17.5 neural progenitors derived from WT and $CB_2^{-/-}$ mice. The number of neurospheres was quantified after 5 consecutive neurosphere passages. Inset: Primary neurosphere generation in the two mouse strains. *B)* Primary neurosphere generation was determined after 7 d of exposure of neural progenitors (black bars) to vehicle (*C*), the CB_2-selective agonists HU-308 or JWH-133 (30 nM) and/or the CB_2-selective antagonist SR144528 (2 μM; SR). $CB_2^{-/-}$ progenitors (gray bars) were also used. *C)* Self-renewal of WT neural progenitors (solid line) incubated as above for five consecutive passages. Self-renewal of CB_2-deficient progenitors in the presence of vehicle is also shown (dashed line). *D)* Quantification of BrdU-positive cells from dissociated neurospheres incubated as above for 16 h. *E)* Quantification of BrdU-positive cells (upper panel) and neurosphere generation (lower panel) of progenitors treated with vehicle (*C*) HU-308 (30 nM) and/or PD98059 (10 μM; PD) and/or LY294,002 (5 μM; LY). *F)* ERK and Akt phosphorylation after progenitor challenge with vehicle (*C*) or HU-308 (alone or in the presence of SR144528) for 15 min (ERK) or 2 min (Akt). Results correspond to 3 (*A*, *C*, *E*, and *F*) or 4 (*B* and *D*) independent experiments. Significantly different from control WT cells: $*P < 0.05$, $**P < 0.01$.

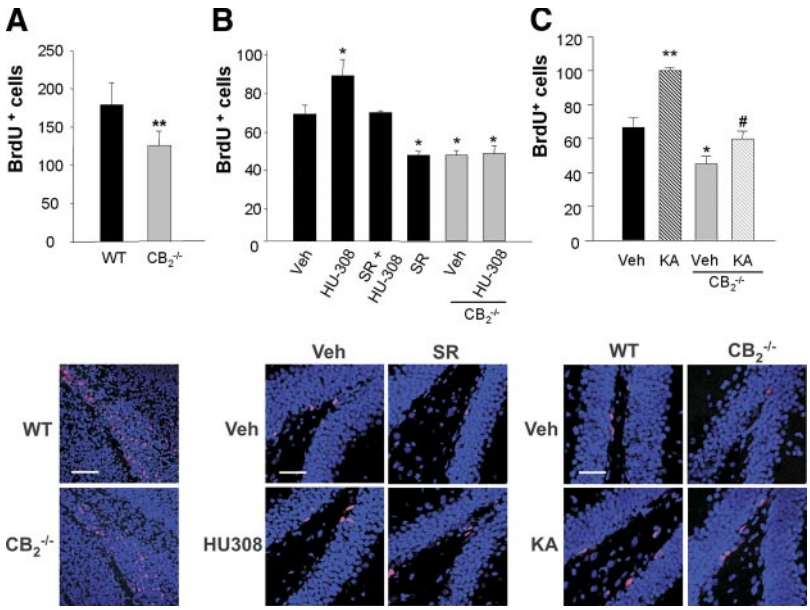

Figure 4. CB_2 receptors control neural progenitor cell proliferation *in vivo*. *A)* Number of BrdU-positive cells per section in the dentate gyrus of WT ($n=5$) and $CB_2^{-/-}$ ($n=7$) mouse E17.5 embryos. *B)* Number of BrdU-positive cells per section in the dentate gyrus of WT (black bars; $n=4$) and $CB_2^{-/-}$ (gray bars; $n=3$) adult mice injected with the indicated agents. *C)* Number of BrdU-positive cells per section in the dentate gyrus of WT (black bars; $n=4$) and $CB_2^{-/-}$ (gray bars; $n=4$) adult mice injected with saline (plain bars) or kainic acid (dashed bars). Lower panels show representative immunostainings of BrdU-positive cells (red) costained with TOTO-3 (blue). Scale bars: 90 μm (*A*) and 45 μm (*B*, *C*). Significantly different from control WT mice: $^{*}P < 0.05$, $^{**}P < 0.01$. Significantly different from WT mice treated with kainic acid: $^{\#}P < 0.05$.

selective receptor ligands. Thus, the CB_2-selective agonists HU-308 (23) and JWH-133 (24) increased both primary neurosphere generation (Fig. 3*B*) and neural progenitor self-renewal (Fig. 3*C*), and both actions were prevented by the CB_2-selective antagonist SR144528. The selectivity of CB_2 agonists was confirmed by the observation that neither HU-308 nor JWH-133 could enhance neurosphere generation in CB_2-deficient neural progenitors (Fig. 3*B*). Moreover, HU-308 and JWH-133 increased the number of BrdU-incorporating cells in a CB_2-dependent manner (Fig. 3*D*), supporting the direct impact of CB_2 receptor activation on neural progenitor cell proliferation. Likewise, increased neurosphere generation was observed on CB_2 receptor activation in postnatal and adult progenitors (percentage of neurosphere number relative to vehicle incubations: HU-308: 130±8% and 161±20%, respectively; JWH-133: 154±22% and 149±6%, respectively), and this action was prevented by SR144528 (data not shown).

To determine the potential signaling mechanism responsible for CB_2-mediated proliferation, neural progenitors were incubated in the presence of HU-308 and selective inhibitors of the ERK cascade (PD98059) and the phosphatidylinositol 3-kinase/Akt pathway (LY294,002). HU-308 induction of cell proliferation was prevented by both inhibitors (Fig. 3*E*, upper panel), a finding that was confirmed in neurosphere generation assays (Fig. 3*E*, lower panel). These results prompted us to analyze CB_2-mediated regulation of ERK and Akt. Thus, HU-308 stimulated ERK and Akt, and this action was prevented by SR144528 (Fig. 3*F*).

CB_2 receptors control neural progenitor cell proliferation *in vivo*

The functional relevance of the CB_2 receptor in controlling neural progenitor cell proliferation *in vivo* was determined by assessing BrdU incorporation in CB_2-deficient mice and their WT littermates. In both embryonic (**Fig. 4*A***) and adult (Fig. 4*C*) brain, CB_2 knockout animals showed a significant decrease in BrdU-labeled cells in the dentate gyrus of the hippocampus. These results suggest that neural progenitor proliferation *in vivo* may be suitable for CB_2 pharmacological manipulation. Thus, HU-308 and/or SR144528 were administered for 5 consecutive days and hippocampal proliferation was determined. Importantly, CB_2 activation increased progenitor proliferation, while CB_2 blockade exerted the opposite action (Fig. 4*B*). The selectivity of HU-308 *in vivo* was confirmed by SR144528 antagonism and by the lack of HU-308 agonistic effect in CB_2-deficient mice. We further tested whether CB_2 receptors may be implicated in the control of neural progenitor cell proliferation in a situation of brain injury such as kainate-induced excitotoxicity. As shown in Fig. 4*C*, the remarkable excitotoxic stimulation of neural progenitor cell proliferation was abrogated in CB_2-deficient mice.

DISCUSSION

To date, the effects of endocannabinoids on the modulation of synaptic plasticity and neuronal excitability (4), as well as of neural cell survival (25, 26), have been attributed solely to the engagement of "central" CB_1 receptors. The expression pattern of the CB_1 receptor is regulated during brain development (27), and the receptor remains expressed at high levels in differentiated neurons and at lower levels in glial cells of various adult brain areas, such as the hippocampus, basal ganglia, and cortex (3, 4). In contrast, the presence of the "peripheral" CB_2 receptor in differentiated neurons and glial cells is more restricted (4). Thus, only recently the expression of CB_2 receptors in normal brain could be demonstrated in the cerebellum (13, 14) as well as in a subpopulation of neurons of the

vagus nerve in the brainstem (12), where it participates in the regulation of emesis. In addition, CB_2 receptor expression in the brain is also found in microglia (9, 10) and endothelial cells (11). Here, we provide evidence that neural progenitors from embryonic to adult stages express functional CB_2 receptors. Of interest, other studies had previously suggested an inverse relation between CB_2 receptor expression and stage of cell differentiation. For example, CB_2 receptor expression decreases during B-cell differentiation (28) and increases with dedifferentiation (i.e., with increased malignancy) of glial tumors (29). Likewise, CB_2 receptor activation and overexpression (30) block neutrophil cell differentiation. Thus, it is tempting to speculate that endocannabinoids may control neural progenitor cell function via CB_2 receptors acting as a "cell dedifferentiation signal" by favoring a nondifferentiated, proliferative state.

During mammalian development, the generation of the CNS relies on a finely regulated balance of neural progenitor proliferation, differentiation and survival that is controlled by a number of extracellular signaling cues (19, 20). The existence of hippocampal neurogenesis in the adult brain has received strong support by the identification of a neural progenitor cell population located in the subgranular zone (19, 20, 22). These neural progenitors give rise to newly generated cells that can integrate properly in hippocampal circuits and thus may contribute to synaptic plasticity (31), cognitive functions (20), and neuroregeneration on brain damage (32). Our finding of impaired neural progenitor proliferation after neuroexcitotoxic damage in CB_2-deficient mice, together with the protective role of endocannabinoids in a variety of brain damage models (25), suggest that endocannabinoids generated on demand on brain injury may enhance neural progenitor proliferation via CB_2 receptors. The relevance of our results is further strengthened by the recent demonstration of the role of the endocannabinoid system in the regulation of adult neurogenesis. Hippocampal progenitors produce endocannabinoids in a regulated manner and express the CB_1 receptor (16). *In vivo* regulation of cannabinoid signaling during CNS development alters neuronal activity (33) and generation (15, 34). These findings add to the reported impairment of cognitive functions in CB_1 knockout mice (35) and the potential of cannabinoid-mediated regulation of adult neurogenesis (17, 36).

The use of cannabinoids in medicine is severely limited by their well-known psychotropic effects. Although psychoactivity tends to disappear with tolerance on continuous cannabinoid use (4), it is obvious that cannabinoid-based therapies devoid of side effects would be desirable. As the unwanted effects of cannabinoids are mediated largely or entirely by CB_1 receptors within the brain (4), the most conceivable possibility would be to use cannabinoids that selectively target CB_2 receptors. In this context, the recent synthesis of CB_2-selective agonists (23, 24) opens an attractive clinical possibility. By showing that CB_2 receptor activation is functional in stimulating neural progenitor cell proliferation *in vitro* and *in vivo*, the present report, together with the implication of CB_2 receptors in the control of processes such as pain initiation (37, 38), emesis (12), neuroinflammation (39, 40), and brain-tumor cell death (29) opens the attractive possibility of finding cannabinoid-based therapeutic strategies for neural disorders devoid of nondesired psychotropic effects. Specifically, the proliferative effect of cannabinoids reported here may set the basis for the potential pharmacological modulation of neural progenitor cell fate by CB_2-selective ligands. FJ

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Non-psychoactive CB_2 cannabinoid agonists stimulate neural progenitor proliferation

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SPECIFIC AIMS

Endocannabinoids, the endogenous counterparts of the bioactive components produced by marijuana (*Cannabis sativa*), are generated on demand as a consequence of brain injury and they exert a neuroprotective action. In addition, endocannabinoids target neural progenitor (NP) cells and regulate cell proliferation and differentiation via the seven-transmembrane CB_1 receptor. However, the pharmacological manipulation of NPs by cannabinoids is hampered by the typical marijuana-like CB_1-mediated psychoactive effects. In contrast to the wide expression of CB_1 in the brain and many other organs, cannabinoid receptor CB_2 is restrictedly expressed in brain cells while abundant in the immune system. The aim of the present work was to examine whether progenitor cells express the non-psychoactive CB_2 receptor and to study its potential involvement in NP cell fate both in normal and injured brain.

PRINCIPAL FINDINGS

1. Neural progenitors express CB_2 receptors *in vitro* and *in vivo*

Clonally expanded neurospheres derived from different embryonic stages and adult brain were used to determine whether NP cells express CB_2 receptors. RT-polymerase chain reaction (RT-PCR) and Western blot analyses revealed that NPs express CB_2 receptors during development and that its presence remains evident in adult-derived cells. These findings were corroborated in the human neural stem cell line hNSC1. Immnunofluorescence studies with antibodies directed against the CB_2 receptor and markers for multipotent neuroepithelial (nestin), proliferating (bromodeoxyuridine and phosphorylated vimentin), and radial progenitor (RC2 and vimentin) cells confirmed that NPs, including those actively dividing, express CB_2 receptors. Likewise, CB_2 receptors were present in adult brain progenitors. CB_2 expression during neural differentiation was analyzed by RT-PCR and revealed its disappearance during neural differentiation with the concomitant induction of the neuronal (β-tubulin-III) and astroglial markers (GFAP).

Next we determined by confocal microscopy whether CB_2 receptors are expressed *in vivo* in progenitor cells resident in the subgranular zone of the dentate gyrus of the hippocampus, one of the most prominent neurogenic areas throughout life span, including adulthood. CB_2 receptor expression was found only in nestin-positive cells, while we could not find its presence in differentiated hippocampal neurons (NeuN-positive cells) and astrocytes (GFAP-positive cells).

2. CB_2 receptors control neural progenitor cell proliferation and neurosphere generation in vitro

To determine whether CB_2 receptors control NP cell function, we generated neurospheres from CB_2-deficient mice and their wild-type (WT) littermates. Genetic ablation of the CB_2 receptor impaired primary neurosphere generation (**Fig. 1*A***, inset). Moreover, NP self-renewal, as determined by neurosphere generation for several consecutive passages, was reduced in CB_2-deficient cells (Fig. 1*A*). The observed impairment of NP function in $CB_2^{-/-}$ cell cultures prompted us to analyze the prominin (cluster of differentiation-133)-positive subpopulation, as these cells are considered the stem cell fraction responsible for neurosphere formation activity. Of interest, $CB_2^{-/-}$ neurospheres, when compared to WT cultures by flow cytometry analysis, showed a reduction in their cluster of differentiation (CD)-133^+ subpopulation (cluster of differentiation-133^+ cells: $5.8\pm2.0\%$ *vs.* $7.4\pm1.5\%$, respectively).

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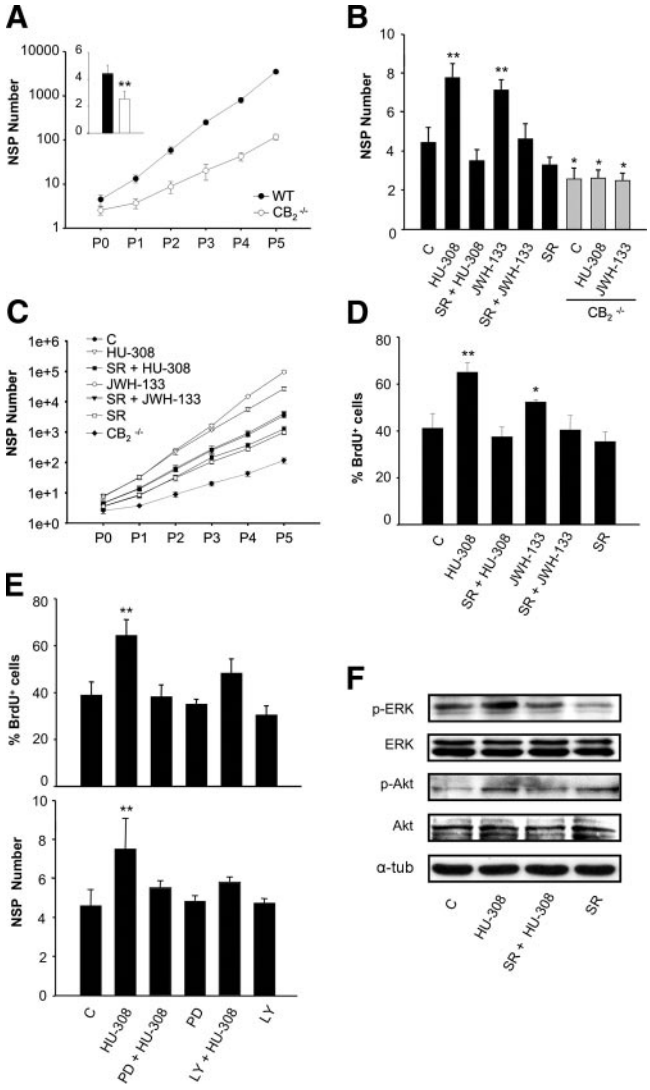

Figure 1. CB_2 receptors control neurosphere generation and neural progenitor cell proliferation *in vitro. A)* Self-renewal of E17.5 neural progenitors derived from WT and $CB_2^{-/-}$ mice. The number of neurospheres was quantified after 5 consecutive neurosphere passages. *Inset*) Primary neurosphere generation in the two mouse strains. *B)* Primary neurosphere generation was determined after 7 d of exposure of neural progenitors (black bars) to vehicle (*C*), the CB_2-selective agonists HU-308 or JWH-133 (30 nM) and/or the CB_2-selective antagonist SR144528 (2 μM; SR). $CB_2^{-/-}$ progenitors (gray bars) were also employed. *C)* Self-renewal of WT neural progenitors (solid line) incubated as above for 5 consecutive passages. Self-renewal of CB_2-deficient progenitors in the presence of vehicle is also shown (dashed line). *D)* Quantification of BrdU-positive cells from dissociated neurospheres incubated as above for 16 h. *E)* Quantification of BrdU-positive cells (upper panel) and neurosphere generation (lower panel) of progenitors treated with vehicle (*C*), HU-308 (30 nM) and/or PD98059 (10 μM; PD) and/or LY294,002 (5 μM; LY). *F)* ERK and Akt phosphorylation after progenitor challenge with vehicle (*C*) or HU-308 (alone or in the presence of SR144528) for 15 min (ERK) or 2 min (Akt). Results correspond to 3 (*A*, *C*, *E*, and *F*) or 4 (*B* and *D*) independent experiments. Significantly different from control WT cells: $^*P < 0.05$, $^{**}P < 0.01$.

The functional relevance of the CB_2 receptor was evaluated by incubating neurospheres with the CB_2-selective agonists HU-308 and JWH-133, both of which increased neurosphere generation (Fig. 1*B*) and NP self-renewal (Fig. 1*C*). These actions were prevented by the CB_2-selective antagonist SR144528. The selectivity of the CB_2 agonists was confirmed in CB_2-deficient NPs, in which HU-308 and JWH-133 were unable to enhance neurosphere generation (Fig. 1*B*). Moreover, HU-308 and JWH-133 increased the number of bromodeoxyuridine (BrdU)-incorporating cells in a CB_2-dependent manner (Fig. 1*D*), supporting the direct impact of CB_2 receptor activation on cell proliferation. Likewise, increased neurosphere generation was observed on CB_2 activation in postnatal and adult progenitors (data not shown).

To determine the potential signaling mechanism responsible for CB_2-mediated proliferation, neural progenitors were incubated with HU-308 and selective inhibitors of the extracellular signal-regulated kinase (ERK) cascade (PD98059) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (LY294,002). HU-308 induction of cell proliferation was prevented by both inhibitors (Fig. 1*E*, upper panel), a finding that was confirmed in neurosphere generation assays (Fig. 1*E*, lower panel). These results prompted us to analyze CB_2-mediated regulation of ERK and Akt. Thus, HU-308 stimulated ERK and Akt, and this action was prevented by SR144528 (Fig. 1*F*).

3. CB_2 receptors control neural progenitor cell proliferation *in vivo*

The functional relevance of the CB_2 receptor in controlling neural progenitor cell proliferation *in vivo* was determined by assessing BrdU incorporation in CB_2-deficient mice and their WT littermates. In both embryonic (**Fig. 2*A***) and adult (Fig. 2*C*) brain, CB_2 knock-

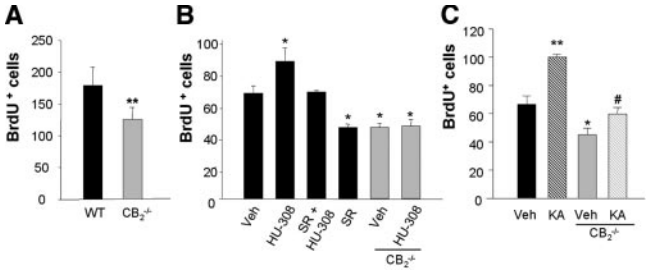

Figure 2. CB_2 receptors control neural progenitor cell proliferation *in vivo. A)* Number of BrdU-positive cells per section in the dentate gyrus of WT (n=5) and $CB_2^{-/-}$ (n=7) mouse E17.5 embryos. *B)* Number of BrdU-positive cells per section in the dentate gyrus of WT (black bars; n=4) and $CB_2^{-/-}$ (gray bars; n=3) adult mice injected with the indicated agents. *C)* Number of BrdU-positive cells per section in the dentate gyrus of WT (black bars; n=4) and $CB_2^{-/-}$ (gray bars; n=4) adult mice injected with saline (plain bars) or kainic acid (dashed bars). Significantly different from controls: $^*P < 0.05$, $^{**}P < 0.01$. Significantly different from WT mice treated with kainic acid: $^{\#}P < 0.05$.

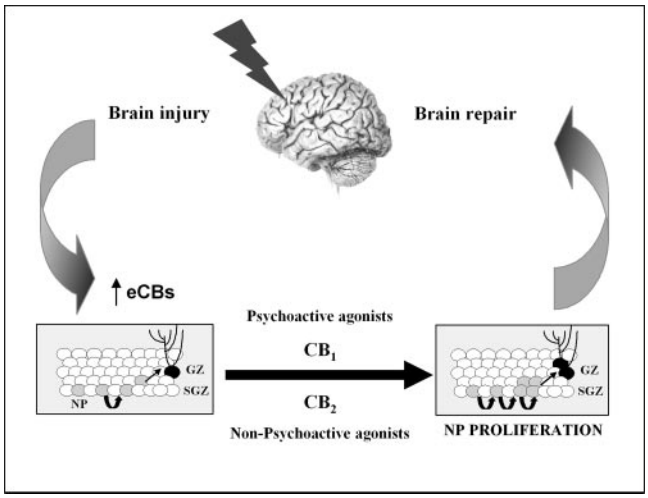

Figure 3. Schematic diagram showing cannabinoid action on hippocampal neural progenitor (NP) proliferation and its potential involvement in the regenerative response to brain injury.

out animals showed a significant decrease in BrdU-labeled cells in the dentate gyrus of the hippocampus. These results suggest that neural progenitor proliferation *in vivo* may be suitable for CB_2 pharmacological manipulation. Thus, HU-308 and/or SR144528 were administered for 5 consecutive days and hippocampal proliferation was determined. Importantly, CB_2 activation increased progenitor proliferation, while CB_2 blockade exerted the opposite action (Fig. 2*B*). The selectivity of HU-308 *in vivo* was confirmed by SR144528 antagonism and the lack of HU-308 agonistic effect in CB_2-deficient mice. We further tested whether CB_2 receptors may be implicated in the control of neural progenitor cell proliferation in a situation of brain injury such as kainate-induced excitotoxicity. As shown in Fig. 2*C*, the remarkable excitotoxic stimulation of neural progenitor cell proliferation was abrogated in CB_2-deficient mice.

CONCLUSIONS AND SIGNIFICANCE

The expression pattern of the CB_1 receptor is tightly regulated during brain development and remains expressed at high levels in differentiated neurons and at lower levels in glial cells of various adult brain areas such as the hippocampus, basal ganglia, and cortex. In contrast, the presence of the "peripheral" CB_2 receptor in differentiated neurons is more restricted. Thus, only recently the expression of CB_2 receptors in normal brain could be demonstrated in the cerebellum and in a subpopulation of neurons of the brainstem that participates in emesis regulation. In addition, CB_2 receptor expression in the brain is also found in microglia and endothelial cells. Here, we show that CB_2 cannabinoid receptors are expressed in NP cells both during development and in the adulthood and become down-regulated during their neural cell differentiation. Of interest, other studies previously suggested an inverse relation between CB_2 receptor expression and stage of cell differentiation. For example, CB_2 receptor expression decreases during B-cell differentiation and increases with dedifferentiation (*i.e.*, with increased malignancy) of glial tumors. Thus, it is tempting to speculate that endocannabinoids may control NP cell function via CB_2 receptors acting as a "cell dedifferentiation signal" by favoring a nondifferentiated, proliferative state.

During mammalian development, the generation of the central nervous system (CNS) relies on a finely regulated balance of NP proliferation, differentiation, and survival controlled by a number of extracellular signaling cues. In addition, adult NPs give rise to newly generated cells that can integrate properly in hippocampal circuits and thus may contribute to synaptic plasticity, cognition, or neuroregeneration on brain damage. Our finding of impaired NP proliferation after neuroexcitotoxic damage in CB_2-deficient mice, together with the protective role of endocannabinoids in a variety of brain damage models, suggest that endocannabinoids generated on demand with brain injury may enhance NP proliferation via CB_2 receptors (**Fig. 3**). The relevance of our results is strengthened by the recent demonstration of the role of the endocannabinoid system in the regulation of adult neurogenesis. Hippocampal progenitors produce endocannabinoids and express CB_1 receptors that regulate cell proliferation and neural differentiation. Moreover, *in vivo* regulation of cannabinoid signaling during CNS development alters neuronal activity and generation, events that add to the reported impairment of cognitive functions in CB_1 knockout mice.

The therapeutic use of cannabinoids is severely limited by their well-known psychotropic effects that are mediated by CB_1 receptors within the brain. Thus, for the development of cannabinoid-based therapies devoid of side effects, the most conceivable possibility would be to selectively target CB_2 receptors. In this context, the recent synthesis of CB_2-selective agonists opens an attractive clinical possibility. The finding that CB_2 receptor activation is functional in stimulating NP cell proliferation *in vitro* and *in vivo*, together with the implication of CB_2 receptors in the control of processes such as pain initiation, emesis, neuroinflammation, and brain-tumor cell death, opens the attractive possibility of cannabinoid-based therapeutic strategies for neural disorders devoid of nondesired psychotropic effects. Specifically, the proliferative effect of cannabinoids reported here may set the basis for the potential pharmacological modulation of NP cell fate by CB_2-selective ligands. FJ