Cannabidiol Induces Intracellular Calcium Elevation and Cytotoxicity in Oligodendrocytes

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ABSTRACT

Heavy marijuana use has been linked to white matter histological alterations. However, the impact of cannabis constituents on oligodendroglial pathophysiology remains poorly understood. Here, we investigated the in vitro effects of cannabidiol, the main nonpsychoactive marijuana component, on oligodendrocytes. Exposure to cannabidiol induced an intracellular Ca\(^{2+}\) rise in optic nerve oligodendrocytes that was not primarily mediated by entry from the extracellular space, nor by interactions with ryanodine or IP\(_3\) receptors. Application of the mitochondrial protonophore carbonylcyanide-\(p\)-trifluoromethoxyphenylhydrazone (FCCP; 1 \(\mu\)M) completely prevented subsequent cannabidiol-induced Ca\(^{2+}\) responses. Conversely, the increase in cytosolic Ca\(^{2+}\) levels elicited by FCCP was reduced after previous exposure to cannabidiol, further suggesting that the mitochondria acts as the source of cannabidiol-evoked Ca\(^{2+}\) rise in oligodendrocytes. In addition, brief exposure to cannabidiol (100 nM–10 \(\mu\)M) led to a concentration-dependent decrease of oligodendroglial viability that was not prevented by antagonists of CB\(_1\) and CB\(_2\), vanilloid, A\(_{2A}\) or PPAR\(\gamma\) receptors, but was instead reduced in the absence of extracellular Ca\(^{2+}\). The oligodendrotoxic effect of cannabidiol was partially blocked by inhibitors of caspase-3, -8 and -9, PARP-1 and calpains, suggesting the activation of caspase-dependent and -independent death pathways. Cannabidiol also elicited a concentration-dependent alteration of mitochondrial membrane potential, and an increase in reactive oxygen species (ROS) that was reduced in the absence of extracellular Ca\(^{2+}\). Finally, cannabidiol-induced cytotoxicity was partially prevented by the ROS scavenger trolox. Together, these results suggest that cannabidiol causes intracellular Ca\(^{2+}\) dysregulation which can lead to oligodendrocytes demise.

INTRODUCTION

The hemp plant (Cannabis sativa, marijuana) is the most widely used illicit drug worldwide. Most cannabis users first experience it in adolescence, with nearly half of people aged >12 years in the USA having tried it at least once during their lifetime (SAMSHA, 2007). Adolescence is a critical period for neurodevelopment that includes mechanisms such as the formation or remodeling of axonal projections and the myelination of white matter tracts (Giorgio et al., 2008). Recently, the development of novel magnetic resonance techniques providing sensitive means to detect white matter pathology has allowed to monitor structural abnormalities suggestive of impaired myelination in several white matter tracts of adolescent and young adult cannabis consumers (Arnone et al., 2008; Ashtari et al., 2009; Bava et al., 2009). These data are indicative that marijuana constituents might disrupt the mechanisms involved in the formation and maintenance of the myelin sheath.

Marijuana contains about 80 terpenophenolic constituents named cannabinoids, responsible for the effects of cannabis derivatives on brain function. The main psychoactive component of marijuana, (−)-\(\Delta^9\)-tetrahydrocannabinol (\(\Delta^9\)-THC), exerts most of its biological activity via interaction with two G protein-coupled receptor proteins known as CB\(_1\) and CB\(_2\) cannabinoid receptors (Izzo et al., 2009). Nevertheless, cannabis preparations contain several other bioactive cannabinoids that exert multiple effects via different mechanisms, often independent of CB\(_1\) and CB\(_2\) receptors. Among these compounds, (−)-cannabidiol (CBD) is the most abundant in cannabis extracts, and the one that displays the widest range of pharmacological actions. CBD exhibits anti-inflammatory, analgesic, antioxidant, and neuroprotective effects, which make it a promising agent for the treatment of different pathological conditions, including demyelinating diseases (Pryce and Baker, 2005). CBD has also been shown to induce apoptosis in tumor cells via mechanisms that are cell type dependent and that include the dysregulation of Ca\(^{2+}\) homeostasis and the production of reactive oxygen species (ROS) (Ligresti et al., 2006; Massi et al., 2004; McKallip et al., 2006). Targets for the pharmacological actions of CBD include the modulation of transient receptor potential potential vanilloid 1 channels (TRPV1), 5-HT\(_{1A}\) receptors, glycine receptors, adenosine membrane transporter and A\(_{2A}\) receptors, peroxisome proliferator-activated receptor-\(\gamma\) (PPAR-\(\gamma\)), CB\(_2\) receptors and the endocannabinoid...
degrading enzyme fatty acid amid hydrolase, among others (Izzo et al., 2009).

A limited number of studies have addressed the effects of cannabis constituents in oligodendrocytes (OLs), the cells responsible for the formation of the myelin sheath in the central nervous system. Recently, we reported that Δ⁴-THC reduces the Ca²⁺ transients elicited in OLs by membrane depolarization, via both CB₁ receptor-dependent and -independent mechanisms (Mato et al., 2009). In this study, we sought to investigate the effects of CBD on oligodendroglial Ca²⁺ homeostasis and viability. We report that CBD induces a concentration-dependent elevation of intracellular Ca²⁺ together with a disruption of mitochondrial membrane potential (MMP) and an increase of ROS production, that culminate in a reduction in oligodendroglial viability mediated by the activation of caspase-dependent and -independent death cascades.

**MATERIALS AND METHODS**

**Drugs**

(−)-Cannabidiol (CBD), AM251, AM630, and GW9662 were supplied by Tocris Bioscience (Bristol, UK), resuspended in dimethylsulfoxide (DMSO) to final 10 mM stock solutions and stored at −20°C until use. The caspase-3, -8/6, and -9 specific inhibitors Ac-DEVD-CHO, Ac-LEHD-CHO, and Ac-IETD-CHO were purchased from Peptides International (Louisville, KY). The PARP-1 inhibitor DPQ, the antioxidant compound trolox, and the IP₃ receptor antagonist 2-APB were obtained from Calbiochem (La Jolla, CA). Ryanodine was purchased from Ascent Scientific (Bristol, UK), and carbonylycyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was obtained from Sigma-Aldrich (Madrid, Spain). Other chemicals were from the highest commercial grade available.

**Animals**

Animals were housed in the animal facility of the University of the Basque Country and maintained in a temperature (21°C ± 1°C) and humidity (55% ± 10%) controlled room with a 12 h light-dark cycle. Food and water were available ad libitum. Experiments were carried out in accordance to the guidelines of The European Communities Council Directive 86/609/EEC and were approved by the Animal Research Ethical Committee of our institution.

**Oligodendrocyte Cultures**

OL cultures were prepared from 12-day-old Sprague Dawley rat optic nerves as described previously (Sánchez-Gómez et al., 2003). Cells were seeded into 24-well plates bearing 14-mm-diameter coverslips coated with poly-n-lysine (10 μg/mL) at 10,000 cells per well, and maintained at 37°C and 5% CO₂ in a chemically defined medium.

**Cortical Neuron Cultures**

Cortical neuron cultures were obtained from E18 Sprague-Dawley rat embryos as described elsewhere (Ruiz et al., 2009). Neurons were resuspended in B27 neurobasal medium plus 10% FBS and then seeded onto poly-l-ornithine-coated 48-well plates at 1.5 × 10⁵ cells per well. The medium was replaced by serum-free, B27-supplemented Neurobasal medium 24 h later. The cultures were essentially free of astrocytes and microglia and were maintained at 37°C and 5% CO₂.

**Measurement of [Ca²⁺]ᵢ**

[Ca²⁺]ᵢ was estimated by the 340/380 ratio method as described previously (Alberdi et al., 2010). OLs were loaded with fura-2 AM (5 μM; Invitrogen, Carlsbad, CA) and washed for 5 min in the bathing solution containing 137 mM NaCl, 5.3 mM KCl, 0.4 mM KH₂PO₄, 0.35 mM Na₂HPO₄, 20 mM HEPES, 4 mM NaHCO₃, 10 mM glucose, 1 mM MgCl₂, 2.5 mM CaCl₂, and 0.5 mg/mL BSA, pH 7.4. The Ca²⁺ free solution was prepared by omitting CaCl₂ and adding EGTA 50 μM. Experiments were performed in a coverslip chamber continuously perfused with incubation buffer at 1 mL/min by exposing the cells to CBD in the absence or presence of antagonists. The final concentration of DMSO in the bath solutions was 0.1%, which produced no significant effect on oligodendroglial [Ca²⁺]ᵢ. After each experiment the perfusion system and the recording chamber were carefully washed with diluted ethanol and distilled water to avoid any carryover of CBD. The perfusion chamber was mounted on the stage of a Zeiss (Oberkochen, Germany) inverted epifluorescence microscope (Axiovert 35), equipped with a 150-W xenon lamp Polychrome IV (T.I.L.L. Photonics, Martinsried, Germany) and a Plan Neofluar 40× oil immersion objective (Zeiss). Cells were visualized with a high-resolution digital black/white CCD camera (ORCA, Hamamatsu Photonics Ibérica, Barcelona, Spain), and images were acquired every 5 s. Image acquisition and data analysis were performed using the AquaCosmos software program (Hamamatsu Photonics Ibérica). Results are calculated as area under the curve (AUC) of the response for each cell from the start of CBD application. Data were expressed as the mean ± SEM of at least three independent experiments, and the percentages of inhibition for each condition were always calculated versus control experiments carried out in parallel.

**Cell Viability Assays**

Viability of OLs (1 DIV) and neurons (8 DIV) was determined by 24 h following exposure to CBD (20–30 min). Oligodendroglial viability was assessed using calcein-AM (1 μM; Molecular Probes, Barcelona, Spain), as described previously (Domercq et al., 2010). Viability of neurons was determined by measuring the level of lac-
tate dehydrogenase (LDH; Cytotox 96®, Promega, Madison, WI) released from damaged cells into the culture media. Calcein fluorescence and Citiotox 96® colorimetric reaction were estimated using a Synergy-HT fluorimeter (Bio-Tek Instruments, Beverly, MA). Results were expressed as percentage of cell death versus control, and at least three independent experiments in triplicate were performed for each condition.

**Measurement of Reactive Oxygen Species**

Intracellular generation of ROS was determined using 5-(and-6)-chloromethyl-2’7’-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA). OLs were loaded with CM-H2DCFDA at 30 μM for 30 min following exposure to CBD, and calcein-AM (1 μM) was used to quantify the number of cells within the reading field. Fluorescence was measured using a Synergy-HT fluorimeter, and results were expressed as the mean ± SEM of at least three independent experiments carried out in triplicate.

**Determination of Mitochondrial Membrane Potential**

5,5’,6,6’-Tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodine (JC-1, Invitrogen) was used to measure mitochondrial membrane potential (MMP) in OLs (Domercq et al., 2010). Cells were loaded with JC-1 (1 μM) for 15 min and washed twice. Red/green fluorescence was measured using a Synergy-HT fluorimeter before adding CBD, and at different time points following addition of the drug. The glutamate receptor agonist AMPA was used as control for mitochondrial depolarization in these experiments (Sánchez-Gómez et al., 2003). All experiments were performed in quadruplicate and the values provided (mean ± S.E.M. of at least three independent experiments) are expressed as percentages of change versus control wells.

**Data Analysis**

In Ca²⁺ measurement experiments n corresponds to the number of cells used for each condition, whereas in the rest of assays it corresponds to the number of cultures tested. Data were analyzed with Excel (Microsoft, Seattle, WA) and Prism (GraphPad Software, San Diego, CA) software. Statistical significance between two data sets was tested by using Student’s t-test with a critical probability of P < 0.05.

**RESULTS**

**Cannabidiol Induces Intracellular Ca²⁺ Elevations in Oligodendrocytes**

Exposure of rat optic nerve OLs to CBD evoked a sustained and concentration-dependent elevation of [Ca²⁺], (Fig. 1A). The increase of [Ca²⁺]i induced by CBD (1 μM) was not prevented by previous application of CB₁ (AM281, 1 μM) or CB₂ (AM630, 1 μM) receptor antagonist, nor by the TRMV1 blocker capsazepine (1 μM) (Fig. 1B). Exposure of OLs to a Ca²⁺-free bathing solution induced a sustained decrease of [Ca²⁺]i (data not shown), and only partially prevented the Ca²⁺ response elicited by CBD (1 μM) (42.6% ± 3% inhibition; n = 68 cells, P < 0.0001) (Fig. 1B). Previous reports suggest that influx of Ca²⁺ via voltage-gated and/or store-operated Ca²⁺ channels (VGCCs and SOCCs) could contribute to CBD-induced [Ca²⁺]i elevation in OLs (Mato et al., 2009; Paez et al., 2009). Yet, bath application of the VGCC and SOCC antagonist LaCl₃ (50 μM) did not counteract the increase in [Ca²⁺]i elicited by CBD (Fig. 1B). Similarly, blockade of L-type VGCCs, which mediate the majority of depolarization-induced Ca²⁺ influx in our OL cultures (Mato et al., 2009), with nifedipine (10 μM) did not prevent CBD-induced responses (Fig. 1B). Blockade of ryanodine and IP3 receptors with ryanodine (50 μM) and 2-APB (50 μM) respectively, did not prevent the [Ca²⁺]i increase induced by CBD, suggesting that the endoplasmic reticulum is not involved in CBD-evoked [Ca²⁺]i elevation in OLs (Fig. 1B). Finally, to test the contribution of mitochondria to CBD-induced [Ca²⁺]i responses, we assessed the ability of FCCP, an uncoupler of ATP synthesis that causes mitochondrial Ca²⁺ leakage, to prevent the effect of CBD on [Ca²⁺]i. Bath application of FCCP (1 μM) elicited a sustained elevation in [Ca²⁺]i and completely abrogated the [Ca²⁺]i increase evoked by CBD (Fig. 1C,E). We then reasoned that if Ca²⁺ release from the mitochondria is the main route for CBD-induced [Ca²⁺]i rise in OLs, preexposure to CBD should reduce the ability of FCCP to elevate cytosolic Ca²⁺. Indeed, bath application of CBD (1 μM) partially prevented subsequent FCCP-induced [Ca²⁺]i responses (24.3% ± 3% inhibition; n = 60 cells, P < 0.0001) (Fig. 1D,E). Together, these data indicate that CBD triggers [Ca²⁺]i elevations in OLs mainly via Ca²⁺ release from mitochondria.

**Cannabidiol Triggers Oligodendroglial Death**

CBD-induced [Ca²⁺]i dysregulation has been proposed to have opposing pathophysiological consequences depending on the cell type, including protection from apoptotic signaling involving mitochondrial [Ca²⁺]i dysfuncation in neurons (Ryan et al., 2009) and activation of apoptosis in transformed cells (Ligresti et al., 2006). Exposure of OLs (1 DIV) to CBD (100 nM–1 μM) for 20–30 min induced a concentration-dependent reduction in cell viability that was not observed in cortical neuron cultures (8 DIV) (Fig. 2A), while activation of AMPA receptors (with AMPA 25 μM together with cyclothiazide 100 μM), used as positive control, was toxic (28.6% ± 8% decrease in neuron viability). Selective blockade of CB₁ receptors (AM281, 1 μM), CB₂ receptors (AM630, 1 μM), TRPV1 receptors (capsazepine, 1 μM), PPARγ receptors (GW9662, 100 nM), or adenosine A₂A receptors (ZM241385, 100 nM) did not prevent CBD toxic-
ity to oligodendrocytes (Fig. 2B). By contrast, and reminiscent of the effect of Ca\(^{2+}\) elimination from the bathing solutions on CBD-induced \([\text{Ca}^{2+}]_i\) responses, oligodendroglial death was partially reduced when cells were exposed to CBD in the absence of extracellular Ca\(^{2+}\) (50.4% ± 18% inhibition; \(n = 5\), \(P < 0.05\)) (Fig. 2B), suggesting that dysregulation of \([\text{Ca}^{2+}]_i\) homeostasis contributes to CBD-induced oligodendroglial damage.

**Role of Caspases, PARP-1, and Calpain in CBD-Induced Oligodendrotoxicity**

In order to investigate the molecular mechanisms of CBD-induced oligodendrotoxicity, we evaluated cell death in the presence of different caspase inhibitors. Selective inhibition of caspase-3 with DEVD (100 \(\mu\)M) significantly reduced the decrease in OL viability triggered by exposure to 100 nM and 1 \(\mu\)M CBD, but it did not prevent the toxic effect of 10 \(\mu\)M CBD (Fig. 3A,B), indicating that the concentration of the cannabinoid compound determines the activation of different death pathways. To further characterize the mechanism of CBD-induced toxicity in OLs, subsequent experiments were carried out using 1 \(\mu\)M CBD. Oligodendroglial damage by CBD (1 \(\mu\)M) was partially prevented by inhibitors of caspases upstream from caspase-3, such as the caspase-8 inhibitor IETD (100 \(\mu\)M) (60.9% ± 16% inhibition, \(n = 6\), \(P < 0.05\)) and the mitochondrial-associated caspase-9 inhibitor LEHD (100 \(\mu\)M) (63.2% ± 14% inhibition, \(n = 4\), \(P < 0.05\)) (Fig. 3B), suggesting that the cytotoxic effect of CBD involves the concomitant activation of extrinsic and intrinsic or mitochondrial apoptotic pathways.

The lack of effect of the caspase-3 inhibitor to completely block CBD-induced OL death may indicate the contribution of caspase-independent apoptotic and/or necrotic death pathways to the cytotoxic effect of the cannabinoid compound. An important activator of caspase-independent cell death is a mitochondrial flavoprotein named apoptosis-induced factor (AIF), which mediates...
chromatin condensation and DNA-fragmentation when translocated to the nucleus (Hong et al., 2004). Release of AIF from the mitochondria can be triggered by different mechanisms, including the activation of poly(ADP-ribose) polymerase-1 (PARP-1). To check whether activation of this nuclear enzyme participates to CBD-induced oligodendroglial death, we used the selective PARP-1 inhibitor DPQ alone, and in combination with the caspase-3 inhibitor DEVD. Preincubation of OLs with DPQ (30 μM) partially prevented the cytotoxic effect of 1 μM CBD (22.6% ± 15% inhibition, n = 4, P < 0.05), but it did not counteract oligodendroglial death by 10 μM CBD (34.8% ± 9% cell death in control vs. 31.3% ± 8% in DPQ, n = 4). Concomitant inhibition of PARP-1 and caspase-3 using both inhibitors almost completely blocked the reduction in oligodendroglial viability induced by 1 μM CBD (99.2% ± 12% inhibition, n = 3, P < 0.01) (Fig. 3B). Finally, an additional mechanism that could contribute to CBD-induced cytotoxicity is the activation of the Ca$^{2+}$-sensitive cysteine protease calpain, which has been implicated in excitotoxic oligodendroglial death (Liu et al., 2002). Pretreatment of OLs with PD150606 (100 μM) also reduced oligodendroglial death by 1 μM CBD (52% ± 20% inhibition, n = 7, P < 0.05) (Fig. 3B), indicating the involvement of calpain in the toxic effect of the cannabinoid compound. Overall, these data suggest the participation of both caspase-dependent and -independent mechanism in CBD-induced oligodendroglial death.

Cannabidiol Exposure Disrupts Mitochondrial Membrane Potential and Increases ROS Generation

Changes in MMP and subsequent permeabilization of the mitochondrial membrane are critical steps in
mitochondrial-dependent cell death (Galluzzi et al., 2009; Ijima et al., 2006). Therefore, to further investigate the participation of the mitochondria in CBD-induced oligodendroglial damage, we examined the effect of the cannabinoid compound on MMP using the voltage-sensitive probe JC-1. Exposure to CBD led to a concentration- and time-dependent alteration of oligodendroglial MMP (Fig. 4A). Thus, CBD (1 μM) evoked a sustained MMP hyperpolarization that was statistically significant at all the times tested. By contrast, the highest concentration of CBD assayed (10 μM) elicited an initial hyperpolarization of the mitochondrial membrane, followed by sustained MMP depolarization. We did not detect significant effects of low CBD (100 nM) on MMP. Application of AMPA (100 μM) plus cyclothiazide (100 μM) as a positive control led to a time-dependent loss of MMP (Fig. 4A), as previously described (Sanchez-Gomez et al., 2003). These data indicate that dysregulation of MMP may contribute to the toxic effect of high CBD concentrations in OLs.

ROS over production has been associated with activation of the mitochondrial apoptotic pathway (Galluzzi et al., 2009). In addition, CBD-induced death of tumor cells has been related to the production of ROS (Ligresti et al., 2006; McKallip et al., 2006). Thus, we decided to examine the participation of ROS in CBD-triggered oligodendroglial death. Exposure of OLs to CBD elicited a concentration-dependent increase in intracellular ROS levels (Fig. 4B). Noteworthy, the effect of CBD (1 μM) on ROS production was Ca²⁺-dependent, as it was significantly reduced when experiments were performed in the absence of extracellular Ca²⁺ (94.6% ± 15% inhibition, n = 5, P < 0.05). Finally, the role of CBD-induced ROS production in oligodendroglial death was further confirmed by examining cell death in the presence of the ROS scavenger trolox. Preincubation of OLs with trolox (100 μM) led to a significant reduction of CBD-induced cell death (35.4% ± 6% inhibition, n = 6, P < 0.05) (Fig. 4C). Overall, these data suggest that Ca²⁺-dependent ROS production participates in CBD-induced oligodendrotoxicity.

DISCUSSION
CBD Induces Intracellular Ca²⁺ Mobilization in OLs

Regulation of [Ca²⁺]i homeostasis plays a critical role in oligodendroglial physiology, with patterns of Ca²⁺ signaling determining whether these cells proliferate, migrate, differentiate and myelinate axons, undergo damage, repair or die (Butt, 2006; Matute, 2010). CBD has been reported to trigger [Ca²⁺]i elevations in different cell types, including primary neurons and transformed cells (Drysdale et al., 2006; Ligresti et al., 2006; Ryan et al., 2009). Exposure to CBD induced a concentration-dependent [Ca²⁺]i response in OLs, in marked contrast to the effects of other cannabinoid compounds in these cells (Mato et al., 2009). Indeed, we have recently shown that high concentrations (3 μM) of different CB1 and/or CB2 receptor agonists, including the psychoactive phytocannabinoid Δ⁹-THC, do not modulate basal [Ca²⁺]i levels in OLs, arguing against the possibility that CB1 and CB2 receptors are involved in CBD-induced [Ca²⁺]i elevations in these cells. Consistently, antagonists of CB1 and CB2 receptors did not counteract the Ca²⁺ response triggered by CBD in OLs, as neither did the blockade of TRPV1 receptors, VGCCs or SOCCs. These last results are in contrast to the observed contribution of VGCCs to the [Ca²⁺]i response triggered by CBD in primary neurons (Drysdale et al., 2006), suggesting that cell type determines the mechanisms of CBD-induced regulation of Ca²⁺ homeostasis.
The fact that CBD-induced \([Ca^{2+}]_i\) signal is not completely abrogated when \(Ca^{2+}\) is eliminated from the bathing solution clearly points to an important contribution of intracellular sources to the effect of the cannabinoid compound. Nevertheless, a role for extracellular \(Ca^{2+}\) cannot be unequivocally concluded from these experiments, as elimination of \(Ca^{2+}\) from the bathing solution induced a sustained decrease of oligodendroglial \([Ca^{2+}]_i\) that might per se modulate CBD-induced \([Ca^{2+}]_i\) response. The high lipophilic nature of cannabinoids grants them direct access to intracellular targets such as the mitochondria, and previous work has shown the direct contribution of this organella to CBD-induced \([Ca^{2+}]_i\) response (Ryan et al., 2009). In agreement with this idea, bath application of the mitochondrial protonophore FCCP, known to elicit \(Ca^{2+}\) release from the mitochondrial compartment, evoked a fast increase in \([Ca^{2+}]_i\), and completely prevented further \(Ca^{2+}\) responses by CBD. In addition, the ability of FCCP to elevate \([Ca^{2+}]_i\), in OLs was significantly reduced by previous application of CBD, suggesting that the cannabinoid compound elicits partial mitochondrial \(Ca^{2+}\) depletion. Finally, CBD-induced \([Ca^{2+}]_i\), elevation was not altered by blockers of ryanodine and IP3 receptors. Altogether, our findings indicate that mitochondria is the main source of the \([Ca^{2+}]_i\) elevations triggered by CBD in OLs.

CBD Triggers Oligodendroglial Death via Caspase-Dependent and -Independent Pathways

Cytosolic \(Ca^{2+}\) overload is involved in a number of pathological conditions that imply oligodendroglial death (Matute, 2010). Short-term exposure of optic nerve OLs to CBD induced a concentration-dependent reduction of cell viability that was not observed in cortical neurons. Our findings in neurons are in agreement with previous reports on the absence of toxic effects of high CBD concentrations in a variety of nontransformed cells (Hampson et al., 1998; Ligresti et al., 2006) and show that OLs in vitro display an unusual vulnerability to this cannabinoid compound. Reminiscent of CBD-induced \([Ca^{2+}]_i\) response in OLs, the cytotoxic effect of CBD in these cells was not prevented by antagonists of CB1, CB2 or TRPV1 receptors, nor by blockade of other reported targets of CBD, such as adenosine A2a and PPARγ receptors (Izzo et al., 2009), but was instead reduced when experiments were performed in a \(Ca^{2+}\)-free culture medium. Altogether, these data suggest that elevation of \([Ca^{2+}]_i\) contributes to CBD-induced oligodendrotoxicity, in agreement with previous data on the mechanisms underlying the deleterious activity of this cannabinoid compound in tumor cells (Ligresti et al., 2006).

From a mechanistic point of view, our data suggest the involvement of caspase-dependent apoptotic pathways in the toxic effect of 100 nM and 1 \(\mu\)M CBD, as the specific caspase-3 inhibitor DEVD effectively reduced the decrease in oligodendroglial viability induced by these concentrations of the cannabinoid compound. By contrast, neither DEVD nor the PARP-1 inhibitor DPQ prevented cell death following exposure to 10 \(\mu\)M CBD, suggesting that high concentrations of CBD activate mainly necrotic pathways. These results suggest that the concentration of CBD determines the relative contribution of different biochemical cascades leading to oligodendroglial death, which might well be related to the different degree of \(Ca^{2+}\) overload, mitochondrial dysfunction and ROS production (discussed later), as previously reported for the molecular events leading to excitotoxicity in OLs (Sánchez-Gómez et al., 2003).

Inhibitors of caspase-8 and -9 were protective against cell death by 1 \(\mu\)M CBD, which indicates that mediators of both the extrinsic and intrinsic apoptotic pathways are activated, and point to an important role of the mitochondria in CBD-induced toxicity. The extrinsic apoptosis pathway involving the recruitment of caspase-8 is triggered via the activation of death receptors such as Fas/CD95 and TNFαR1, whereas mitochondrial apoptosis is triggered by intracellular stimuli such as \(Ca^{2+}\) overload and ROS (Galluzzi et al., 2009; Kaufmann and Hengartner, 2001). Alternatively, the extrinsic apoptosis pathway can be recruited by crosstalk from the mitochondrial pathway, as several studies have demonstrated caspase-8 activation downstream of caspase-3 (Wieder et al., 2001; von Haefen et al., 2003). In this sense, our data \((Ca^{2+}\) overload, involvement of caspase-9, and alteration of MMP) favor a secondary activation of the extrinsic pathway to apoptosis following CBD exposure.

On the other hand, oligodendroglial death in response to CBD (1 \(\mu\)M) was also partially prevented by inhibition of the DNA repair enzyme PARP-1. Activation of PARP-1 is a key event in the cell death pathway induced by AIF, a major mediator of caspase-independent neuronal and oligodendroglial death that is released from the mitochondria upon stimulus that disrupt MMP (Hong et al., 2004; Jurewicz et al., 2005). Finally, CBD-induced oligodendroglial death could also be prevented by inhibition of the \(Ca^{2+}\)-dependent cysteine protease calpain, which can trigger both caspase-dependent and -independent cell death following \(Ca^{2+}\) overload, which has been involved in oligodendroglial excitotoxicity (Liu et al., 2002; Sánchez-Gómez et al., 2003). Altogether, these data suggest that both caspase- and AIF-dependent death pathways contribute to the cytotoxic effect of CBD in oligodendrocytes.

Role of Mitochondrial Dysfunction and ROS Production in CBD-Induced Oligodendrotoxicity

Alterations in mitochondrial structure and function play a key role in the regulation of cell death, and it is generally accepted that changes in mitochondrial membrane integrity precede apoptosis (Galluzzi et al., 2009). Exposure to CBD evoked a concentration- and time-dependent alteration of MMP, with 1 \(\mu\)M inducing a
sustained hyperpolarization, whereas 10 μM induced an initial hyperpolarization and a subsequent depolarization. These results are reminiscent of previous findings showing that MMP dysregulation depends on the intensity and duration of the insult, as both hyperpolarization and depolarization have been reported to occur in response to ischemia in neurons (Ijima, 2006). Previous studies in different cell types suggest that MMP hyperpolarization is an early pivotal point in apoptotic pathways, that can lead to cytochrome c release, activation of executioner caspase-3 (Poppe et al., 2001), and of the PARP-1/AIF cascade (Kim et al., 2003). Recent work from our lab has linked MMP hyperpolarization with damage to OLs in response to lipopolysaccharide-activated microglial cells (Domercq et al., 2007). On the other hand, depolarization of the inner mitochondrial membrane and release of proapoptotic molecules such as cytochrome c and AIF are known features in oligodendroglial damage triggered by different stimuli, including excitotoxicity, ischemia, and exposure to amyloid beta oligomers (Alberdi et al., 2010; Domercq et al., 2010; Sánchez-Gómez et al., 2003). Hyperpolarization of the mitochondrial membrane can occur in response to ROS production (Li et al., 1999; Nagy et al., 2003), and conversely, disruption of MMP may trigger intracellular ROS elevation (Skulachev 2006). Thus, severe mitochondrial dysfunction associated with depolarization and permeabilization of the mitochondrial membrane has been consistently linked to increased ROS levels in different cell types, including OLs (Alberdi et al., 2010; Galluzzi et al., 2009; Sánchez-Gómez et al., 2003). Noteworthy, different cannabinoid compounds have been reported to trigger mitochondrial depolarization, ROS production, and cell death independently of CB receptors (Athanasious et al., 2007). Exposure of OLs to CBD elicited a significant elevation of ROS levels at all the concentrations tested, and the participation of ROS in CBD-induced cytotoxicity, confirmed by the protective effect of the antioxidant trolox, is in agreement with the well-known vulnerability of these cells to the deleterious effects of oxidative stress (Alberdi et al., 2010; Benjamins et al., 2003; Liu et al., 2002; Sánchez-Gómez et al., 2003). Although the ability of CBD to induce ROS production in OLs may seem surprising in view of its antioxidant properties when used at high concentrations (Hampson et al., 1998), here we provide evidence in favor of a possible mechanism responsible for ROS production by CBD in addition to the reported dysregulation of the MMP, which is the elevation of [Ca\(^{2+}\)]. Indeed, CBD-induced intracellular Ca\(^{2+}\) increase occurred in the same range of concentrations triggering ROS production and oligodendrotoxicity. Furthermore, the ability of CBD to generate ROS seems to be Ca\(^{2+}\)-dependent, as it was erased when Ca\(^{2+}\) was eliminated from the culture medium. As a whole, the most obvious interpretation of our findings is that CBD induces mitochondrial dysfunction and oxidative stress ensuing oligodendroglial death, reminiscent of previous reports on the mechanisms underlying the antitumoral effects of this compound (Ligresti et al., 2006; McKallip et al., 2006).

**Physiological Implications**

Recent brain imaging studies have provided evidence that early and/or heavy marijuana use is linked to white matter microstructure abnormalities suggesting regional defective and/or decreased myelination (Arnone et al., 2008; Ashtari et al., 2009; Bava et al., 2009; Matochick et al., 2005). Although the underlying pathophysiological mechanisms remain poorly understood, these findings indicate that cannabis components might dysregulate oligodendroglial physiology and lead to subsequent defects in the maturation of the myelin sheath, which continues until late adolescence. In this context, we have previously reported that activation of CB\(_1\) receptors in OLs by Δ\(^9\)-THC reduces depolarization-induced Ca\(^{2+}\) influx in these cells, with a possible effect in axonal recognition and myelin initiation (Mato et al., 2009). The present results showing that CBD modulates Ca\(^{2+}\) homeostasis and viability of OLs are the first report on the activity of the major nonpsychoactive constituent of cannabis derivatives in these cells. Although the sensitivity of human OLs to the effects of CBD in vivo remains to be determined, an important consideration when evaluating these data is that CBD concentrations above 100 nM have been measured in the plasma of cannabis consumers (Agurell et al., 1986). Our results thus provide an additional mechanism by which cannabis abuse may negatively affect the formation, maintenance, and repair of the myelin sheath.

Although the physiological consequences of white matter abnormalities in cannabis users are unclear, two lines of evidence support the possibility that they contribute to the subsequent development of psychiatric disorders. First, cannabis abuse has been consistently associated to increased risk of psychiatric conditions such schizophrenia (Arseneault et al., 2004; Henquet et al., 2005) and major depression (Bovasso 2001; Medina et al., 2007). Second, accumulating data converge to implicate abnormalities in white matter connectivity, thought to arise from oligodendroglia dysfunction or even death, in the pathogenesis of both schizophrenia and depression (Davis et al., 2003; Fields 2008; Medina et al., 2007). In this regard, our results suggest that CBD-induced dysregulation of oligodendroglial physiology may contribute to the harmful effects of cannabis on mental health.

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**REFERENCES**

Agurell S, Halldin M, Lindgren JE, Ohlsson A, Widman M, Gillespie H, Hollister L. 1986. Pharmacokinetics and metabolism of delta-1-
tetrahydrocannabinol and other cannabinoids with emphasis on man. Pharmacol Rev 38:21–34.


