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Activation of the CB_1 cannabinoid receptor protects cultured mouse spinal neurons against excitotoxicity

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Abstract

Significant advances are being made towards understanding the genetic basis for spinal neurodegenerative diseases, however, effective pharmacotherapy remains elusive. One of the primary theories underlying neuron vulnerability is susceptibility to excitotoxicity. We present for the first time evidence that the activation of the CB_1 cannabinoid receptor effectively modulates kainate toxicity in primary neuronal cultures prepared from mouse spinal cord. Addition of Δ^9-tetrahydrocannabinol to the culture medium attenuated the toxicity produced by kainate. The CB_1 receptors were localized to spinal neurons and astrocytes. The neuroprotective effect was blocked with the CB_1 receptor antagonist, SR141716A, indicating a receptor-mediated effect.

Keywords: Excitotoxicity; In vitro; Cannabinoid receptor; Neuroprotection; Spinal

Within the last decade the discovery of specific cannabinoid receptors, agonists, antagonists, and endogenous agonists has led to resurgence in investigating the therapeutic potential of these compounds [19]. The endogenous agonists, anandamide and 2-arachidonylglycerol (2-AG), are synthesized, interact with specific receptors, and transported and degraded, implicating an important biological system that could be manipulated with pharmacotherapy [9].

There are a number of well-documented and potential therapeutic effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) and related agonists, including anti-emesis, analgesia, anticonvulsant action, immunomodulation and lowered intraocular pressure [14]. Two cannabinoid (CB) receptors have been identified to date. One is located predominantly in the central nervous system (CB_1), whereas the other appears to be primarily in cells of immune origin (CB_2). Both are members of the superfamily of G-protein coupled receptors. The CB_1 receptor appears to be sufficient to mediate many of the known pharmacological effects of the cannabinoids [1].

Both clinically and in experimental models, cannabinoids alleviate symptoms associated with motor diseases of the nervous system [7,30]. CB_1 receptors are expressed in motor systems, including localization on motor neurons in the spinal cord and higher brain regions [13,18,27]. CB_1 receptor activation leads to inhibition of adenylyl cyclase, inhibition of calcium channels and/or activation of G-protein coupled inwardly rectifying potassium channels (reviewed in Ref. [1]). In general, activation of CB_1 receptors results in inhibition of neurotransmitter release, consistent with observed physiological responses [9]. In particular, CB_1 receptor agonists inhibit presynaptic release of glutamate in primary cultures of hippocampal neurons and prevent neuronal cell death in a model of synaptically-mediated excitotoxicity [23]. A synthetic receptor agonist, CP-55,940, was a partial agonist for inhibition of glutamate release, but completely blocked excitotoxicity in this system [23]. These data suggest that excitotoxicity may be prevented by cannabinoids without completely blocking glutamatergic neurotransmission. Drugs that completely block glutamatergic neurotransmission produce serious side effects (PCP-like hallucinations, etc.) [15]. With this in mind, it is intriguing to note that Δ^9-THC is a partial agonist for CB_1 receptor mediated effects in neurons [24,28].

One of the primary theories underlying neurodegenerative diseases is excitotoxicity. Paradigms of excitotoxicity include over-stimulation of NMDA and non-NMDA channels with agonists such as glutamate and kainate [6]. The investigation reported here evaluates the ability of the cannabinoid Δ^9-THC to inhibit neurotoxicity caused by overstimulation of NMDA and non-NMDA channels in the spinal cord. Our data demonstrates a CB_1 receptor-mediated attenuation of kainate toxicity in cultured mouse spinal

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cord neurons. The inhibitory effect of Δ^9-THC, at concentrations relevant to receptor stimulation, was similar to the effects observed with the kainate receptor antagonist 2,3-dihydro-6-nitro-7-sulfamoyl-benz(F)quinoxaline (NBQX).

Mixed spinal cord cultures were prepared from 13 day old mouse embryos using methods previously described by others [5]. Meninges and dorsal root ganglia were removed from spinal cords prior to plating. Cells were plated in Eagle's MEM containing 10% fetal bovine serum, 10% heat-inactivated horse serum and 2 mM glutamine and 25 mM glucose on 12 mm poly-D-lysine coated cover slips at a density of one 'spinal cord' per two wells of a 12-well plate (~$1–2 \times 10^5$ cells/cm^2). Cultures were maintained at 37°C in 5% CO_2. After 1–2 days in vitro (DIV), 10^{-5} M cytosine arabinoside was added for 1–2 days to inhibit non-neuronal cell division. The cells were then cultured in feeding media (Neurobasal Media, LTI, Rockville, MD, supplemented with 2% B27, 2% heat-inactivated horse serum and 2 mM glutamine). Media was replaced every 5 days and experiments were performed 7–15 DIV. This method provides a mixed population of neurons and glia. For astrocyte cultures, cells were plated directly on culture dishes in MEM media, and passaged 1 week later. Under these conditions neurons do not survive, but astrocytes grow [5,21]. Experiments on astrocytes were performed 14 DIV.

CB_1 receptors were visualized using immunocytochemistry in cultured neurons essentially as described [27]. Cells were grown on laminin coated glass coverslips, and fixed in 4% paraformaldehyde in PBS. Fixed coverslip cultures were blocked (1 h, room temperature) in SuperBlock (Pierce, Rockford, IL) in PBS, and incubated (1 h) with an affinity-purified rabbit antibody (1:500 dilution in SuperBlock) directed against the rat CB_1 receptor [27]. The CB_1 receptor antibody was obtained from Dr Ken Mackie (University of Washington) [27]. Slides were then washed in PBS (3×), incubated with FITC-labeled goat anti-rabbit IgG (60 min, 1:32 dilution in PBS, 2.5% Evans Blue, H + L, Cappel, West Chester, PA) at room temperature, rinsed in PBS three times, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Slides were examined with a Nikon IM35 inverted microscope and video camera controlled by the Image 1/Fluor imaging system. For Western immunoblotting, the cells were disrupted by homogenization in PBS. Twenty micrograms of protein was boiled in SDS/β-mercaptoethanol, separated by SDS-PAGE and transferred to Hybond P (Amersham-Pharmacia, Piscataway, NJ). The blot was blocked with 10% non-fat dry milk in TBS (0.9% NaCl, 10 mM Tris, pH 7.4) plus 0.1% Tween-20 for 60 min, incubated overnight at 4°C with primary rabbit CB_1 antibody (Biosource International, Camarillo, CA, 1:400 in blocking buffer, consisting of TBS plus 0.5% Tween-20). Following washing in TBS plus Tween (6×, 3 min each), blots were incubated for 2 h in a 1:10,000 dilution of affinity-purified goat anti-rabbit IgG horseradish peroxidase conjugate (Amersham-Pharmacia, Piscataway, NJ). The blots were washed (6×, 5 min each) in TBS and developed with ECL-Plus (Amersham-Pharmacia, Piscataway, NJ) for 5 min. Blots were apposed to X-ray film for 30 s.

To induce excitotoxicity, cultures were treated overnight in the presence of 100 μM kainic acid. The compounds evaluated were added at the same time as kainate. Δ^9-THC and SR141716A were obtained from NIDA. The final concentration of ethanol was kept constant in the treatments. Cell viability was initially assessed by visual inspection of phase bright neurons. Cell viability was quantitated by a combination of lactate dehydrogenase (LDH) activity and propidium iodide fluorescence. After treatment, 1 ml of cell culture supernatant was transferred into eppendorf tubes (removed media was replaced with 1 ml of fresh media) spun for 1 min to remove non-soluble material, and 100 μl of the supernatant transferred into the corresponding wells of an optically clear 96 well plate. One hundred micro-

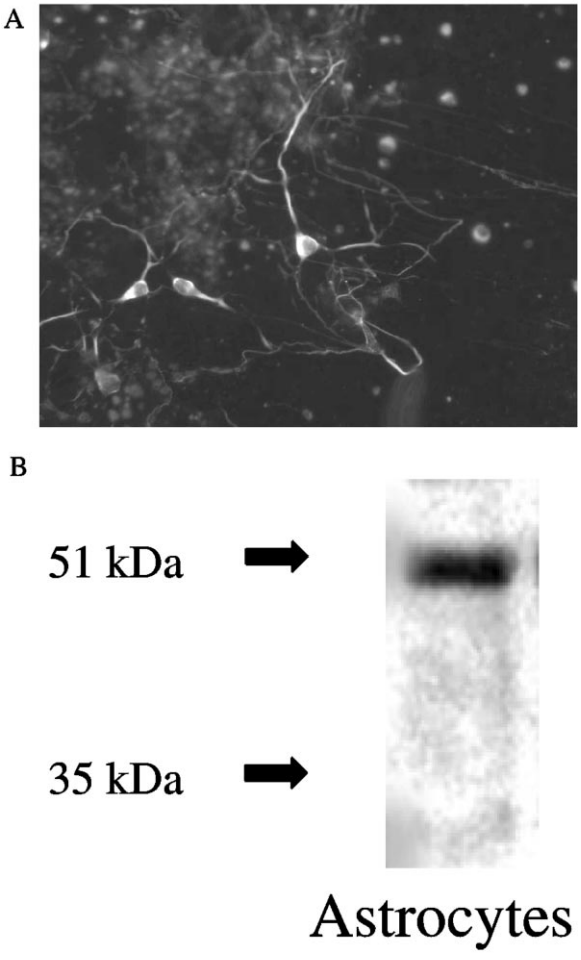

Fig. 1. (A) Immunocytochemistry of primary cultures of mouse spinal cord with anti-CB_1 antibody. Note the strong staining of large neurons, also of smaller neurons, and the diffuse staining of astrocytes and glia. Original magnification 200×. (B) Western immunoblot of spinal cord astrocytes. Astrocytes obtained from mice were probed with anti-CB_1 receptor antibody demonstrating a cross-reacting protein of 51 kDa consistent with the molecular weight of the CB_1 receptor.

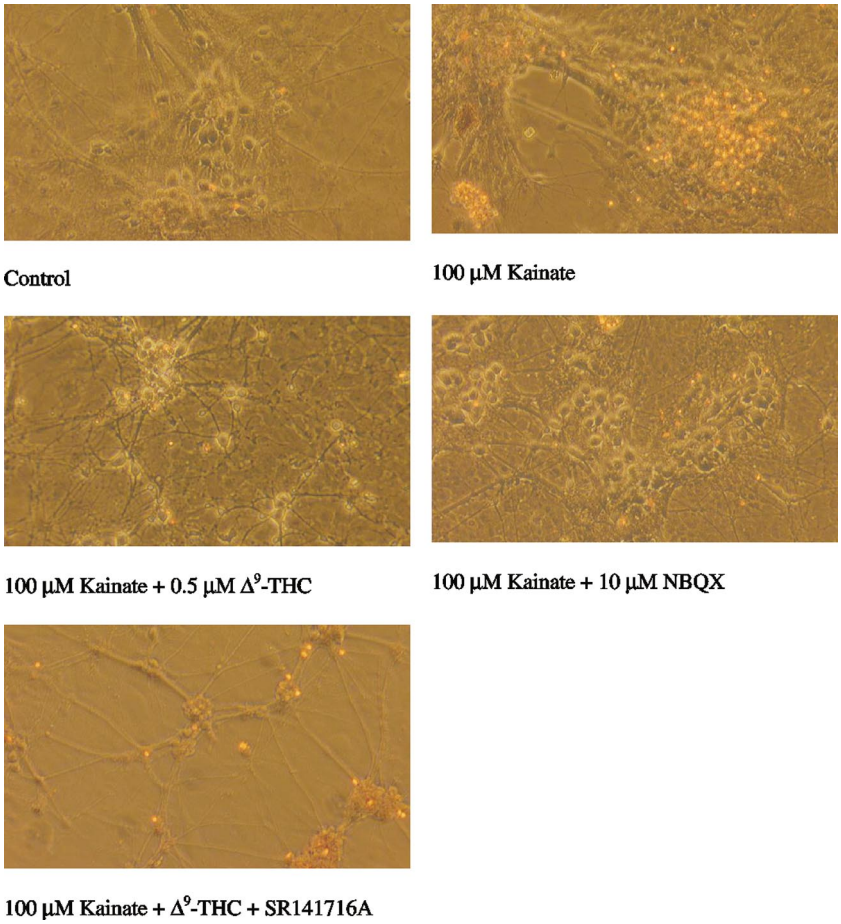

Fig. 2. Representative figure illustrating the efficacy of Δ^9-THC at attenuating toxicity produced by kainate. Primary cultures prepared from spinal cord were treated overnight in the presence of 100 μM kainic acid, kainate plus 0.5 μM Δ^9-THC, kainate plus 10 μM NBQX, kainate plus 0.5 μM Δ^9-THC plus 1 μM SR141716A or vehicle (control). Twenty-four hours later the cultures were evaluated for propidium iodide uptake as a measure of cytotoxicity. The bright field image is superimposed over that of the propidium iodide stained cells. Original magnification 50×.

liters of LDH activity detection mixture (Roche Diagnostics, Mannheim GE) was added and the plates incubated 30 min at 22–25°C. Control wells were lysed with detergent (1% Triton X-100) to get total LDH for a normalization factor. The absorbance of the samples is measured at 490 nm (absorbance of reacted LDH substrate) and 650 nm (overall protein). Cytotoxicity was measured as % cell death relative to total number of cells. Cells were also exposed to a solution of propidium iodide (10 μg/ml) for 10 min at 37°C and then washed with warmed media. Nuclei of dead cells are labeled with propidium iodide and are visible at 485 nM excitation, with fluorescence filtered through a 50 nm band pass filter centered at 580 nm.

To establish a close anatomical relationship between CB_1 receptors, neurons, and glia in mouse spinal cord cultures, receptor localization was evaluated by immunocytochemistry. A representative image of spinal cord neurons stained with an anti-CB_1 receptor antibody is shown in Fig. 1A. This antibody has been well characterized and exhibits high specificity [18,27]. Bright staining was observed in neurons of various sizes. This finding was in agreement with Ong and Mackie [18] who found staining of neurons in both the dorsal and ventral horn in primate spinal cord. In Fig. 1A the very large size and extensive processes of these cells were indicative of motor neurons. Faint staining was observed in astrocytes. To corroborate this, Western analyses were run on astrocyte cultures. A positive band was found at 51 kDa that corresponded to the predicted molecular weight of the cannabinoid receptor (Fig. 1B), as has been described in cortical astrocytes [21]. These data indicated the presence of CB_1 receptors in neurons and astrocytes and provide anatomical rationale for potential neuroprotection by the cannabinoid receptor system.

To evaluate the neuroprotective effects of cannabinoid receptors in brain and spinal cord cultures, we determined whether Δ^9-THC could reduce excitotoxicity produced by direct application of excitatory amino acids. The representative experiment shown in Fig. 2 presents evidence that Δ^9-THC is effective at attenuating toxicity produced by kainate. Primary cultures prepared from spinal cord were treated overnight in the presence of 100 μM kainic acid, 100 μM kainate plus 0.5 μM THC, 100 μM kainate plus 10 μM

NBQX, 100 μM kainate plus 0.5 μM THC plus 1 μM SR141716A, or vehicle (control). Since ethanol was used as a vehicle for THC, and ethanol is an NMDA receptor antagonist, all treatments included the same concentration of ethanol so that it was not a confounding factor in these experiments. Twenty-four hours later the cultures were evaluated for propidium iodide uptake as a visual measure of cytotoxicity. Propidium iodide readily enters and stains non-viable cells [22].

In conjunction with the visual quantification of cell death by propidium iodide uptake, an additional measure of cytotoxicity was employed, LDH release. LDH is a cytoplasmic enzyme that is released upon damage to the plasma membrane. Fig. 3 shows the results of the LDH cytotoxicity assay in mixed spinal cord cultures. The amount of cytotoxicity produced by 100 μM kainate (42%) is expected in a mixed spinal cord culture containing dorsal horn as well as motor neurons [5,29]. We consistently observed attenuation of kainate toxicity by Δ^9-THC in these cultures ($n = 5$). The extent of protection was comparable to that of NBQX, an AMPA/Kainate receptor antagonist. Our experiments indicated the effect of Δ^9-THC was mediated by the cannabinoid CB_1 receptor, as co-addition of the CB_1 receptor antagonist SR141716A blocked the protective effect of Δ^9-THC.

The data presented here indicated that CB_1 receptors are present and expressed in mouse spinal cord neurons and glia. Furthermore, Δ^9-THC attenuates direct excitotoxicity produced by kainic acid in mixed spinal cord cultures. Excitotoxicity is thought to be an important underlying mechanism in the progress of many neurodegenerative diseases [6,16]. Previous reports have demonstrated that cannabinoids produce receptor-mediated inhibition of excitotoxicity at the supraspinal level [23]. However, this investigation is the first to report cannabinoid receptor-mediated attenuation of neuronal excitotoxicity in the spinal cord. This type of inhibition may prove to be useful pharmacotherapy in motor neuron diseases such as amyotrophic lateral sclerosis where neuronal exposure to excess amounts of excitatory amino acids in the spinal cord are thought to be one of the underlying mechanisms of the disease process [16].

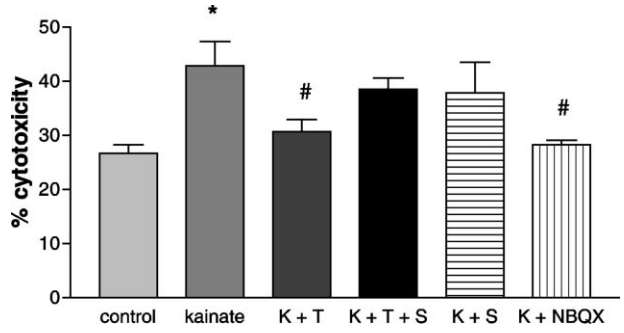

Fig. 3. Protection of kainate mediated cytotoxicity by Δ^9-THC as assessed by LDH release. Neuronal cultures were exposed for 24 h to 100 μM kainate or kainate plus 0.5 μM Δ^9-THC (K + T), kainate plus 0.5 μM Δ^9-THC plus 1 μM SR141716A, the CB_1 receptor antagonist (K + T + S), kainate plus SR141716A alone (K + S), kainate plus 10 μM NBQX, or vehicle (control) ($n = 4$–5 for each condition). *Significant difference ($P < 0.05$) between kainate and control; #significant difference ($P < 0.05$) between treatment and kainate alone using Student's *t*-test.

CB_1 receptors are widely distributed throughout the brain and spinal cord. To date, only a few studies have examined their association with neurological diseases. CB_1 receptor levels are drastically reduced in substantia nigra and lateral globus pallidus in Huntington's Disease [11,20]. The CB_1 receptor antagonist, SR141716A significantly reduced l-dopa-induced dyskinesia in an animal model of Parkinson's as well as in Parkinson's patients [4]. In two animal models of Parkinson's, high levels of anandamide and 2-AG in the globus pallidus are associated with akinesia [8]. CB_1 receptor knockout mice were severely hypoactive in an exploratory test, although their motor coordination was unaltered, suggesting these receptors may be important for initiation of movement [26].

Reports of patients smoking marijuana for relief of spasticity suggest there may be therapeutic benefits from activation of the CB_1 receptor [7]. In a mouse model of multiple sclerosis (chronic relapsing experimental allergic encephalomyelitis), CB_1 and CB_2 receptor agonists quantitatively ameliorate both tremor and spasticity [2]. Recently, Baker et al. demonstrated that endogenous cannabinoids control spasticity in animal models of multiple sclerosis [3].

CB receptor agonists were neuroprotective in excitotoxic cell death in cultured cerebellar neurons [25]. Anandamide/CB receptor agonists decreased hippocampal neuronal loss after transient global cerebral ischemia and reduced infarct volume after permanent focal cerebral ischemia induced by middle cerebral artery occlusion in rats [17]. The same agonists also protected cultured cortical neurons from hypoxia and glucose deprivation, but in contrast to the receptor-mediated neuroprotection observed in vivo, this in vitro effect was not stereoselective and was insensitive to CB_1 and CB_2 receptor antagonists. The authors concluded that cannabinoids may have therapeutic potential in disorders resulting from cerebral ischemia, including stroke, and may protect neurons from injury through a variety of mechanisms.

CB receptor agonists have also been found to possess antioxidant properties, which may contribute to the attenuation of excitotoxicity in primary neuronal cultures [10,12]. This property has not been found to be specifically CB receptor-mediated, as both stereoisomers of 11-OH-Δ^8-THC (HU210 and HU211) exhibit neuroprotective antioxidant properties, and are unaffected by CB_1 receptor antagonist [12].

In hippocampal neurons cannabinoids did not protect against excitotoxicity when glutamate was applied directly but only when excitotoxicity was produced by pre-synaptic release of glutamate [23]. In this study, we found Δ^9-THC could block direct application of the excitotoxic agent kainate. Receptor distribution between the hippocampus and spinal cord may help to explain these differences. In the hippocampus, cannabinoid receptors are primarily located on synaptic terminals [27]. In our mouse spinal

cultures and in primate spinal cord [18] large numbers of CB_1 positive neuronal cell bodies were noted. A cannabinoid receptor mediated post-synaptic inhibition is indicated for the attenuation of excitotoxicity in these cultures.

In conclusion, we have demonstrated the presence of CB_1 receptor immunoreactivity in neurons in primary cultures of mouse spinal cords. We found the CB receptor agonist Δ^9-THC to be effective against toxicity produced by direct addition of kainate to cultured spinal cord neurons. These data suggest that anandamide/CB receptor agonists may slow the progression of excitotoxic cell death.

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