

# Cannabinoid Receptor Agonists Protect Cultured Rat Hippocampal Neurons from Excitotoxicity

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## ABSTRACT

Cannabinoid receptor agonists act presynaptically to inhibit the release of glutamate. Because other drugs with this action are known to reduce excitotoxicity, we tested several cannabimimetics in a model of synaptically mediated neuronal death. Reduction of the extracellular  $Mg^{2+}$  concentration to 0.1 mM evoked a repetitive pattern of intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) spiking that, when maintained for 24 hr, resulted in significant neuronal death. The  $[Ca^{2+}]_i$  spiking and cell death in this model result from excessive activation of *N*-methyl-D-aspartate receptors, as indicated by the inhibition of both  $[Ca^{2+}]_i$  spiking and neuronal death by the *N*-methyl-D-aspartate recep-

tor antagonist CGS19755 (10  $\mu M$ ). The cannabimimetic drug Win55212-2 (100 nM) completely blocked  $[Ca^{2+}]_i$  spiking and prevented neuronal death induced by low extracellular  $Mg^{2+}$  concentrations. These effects on  $[Ca^{2+}]_i$  spiking and viability were stereoselective and were prevented by the CB1 receptor antagonist SR141716 (100 nM). The partial agonist CP55940 (100 nM) also afforded significant protection from excitotoxicity. Cannabimimetic drugs did not protect cells from the direct application of glutamate (30  $\mu M$ ). These data suggest that cannabimimetic drugs may slow the progression of neurodegenerative diseases.

The medicinal use of marijuana is controversial (Annas, 1997). The euphoria produced by  $\Delta^9$ -tetrahydrocannabinol, the principal psychoactive ingredient in marijuana, has led to the widespread recreational use of this drug. Therefore, the potential for abuse must be weighed against the therapeutic benefits of cannabimimetics. These drugs have been reported to be effective in the treatment of chronic pain, glaucoma, epilepsy, and the wasting and emesis associated with acquired immunodeficiency syndrome and cancer chemotherapy (Plasse *et al.*, 1991; Howlett, 1995; Abood and Martin, 1996; Adams and Martin, 1996). The *in vitro* studies described in this report suggest additional therapeutic applications for cannabimimetic drugs.

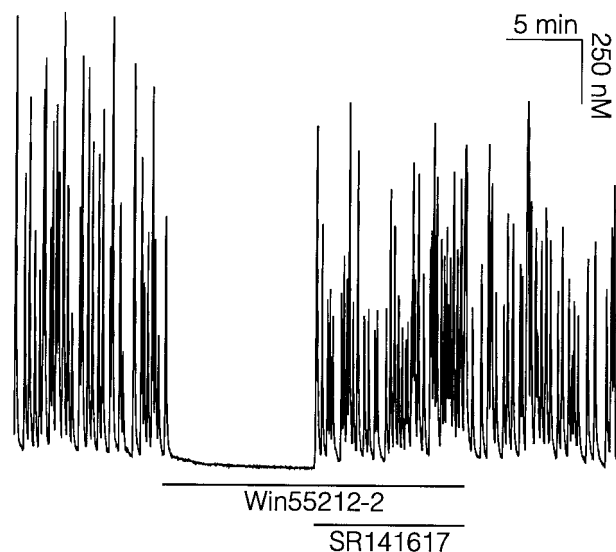
Cannabinoid receptors are distributed throughout the central nervous system (Herkenham *et al.*, 1990) and are present at high densities on the presynaptic terminals of glutamatergic synapses (Twitchell *et al.*, 1997). We previously reported that the activation of these receptors attenuates glutamatergic neurotransmission by acting presynaptically to

inhibit the release of glutamate (Shen *et al.*, 1996). Presumably, activated CB1 receptors, acting via inhibitory G proteins (Childers *et al.*, 1993), activate  $K^+$  channels (Deadwyler *et al.*, 1993; Henry and Chavkin, 1995; Mackie *et al.*, 1995) and inhibit  $Ca^{2+}$  channels (Mackie and Hille, 1992; Twitchell *et al.*, 1997; Shen and Thayer, 1998), resulting in reductions in the release of neurotransmitter. Drugs that attenuate glutamatergic synaptic transmission show promise for the treatment of neurodegenerative disorders (Meldrum, 1993; Choi, 1994; Rothman and Olney, 1995). Several drugs that protect cells from excitotoxic neuronal death have a presynaptic component to their actions, including those that activate adenosine receptors (Abele *et al.*, 1990; Thompson *et al.*, 1992), activate  $K^+$  channels (Abele and Miller, 1990), and inhibit voltage-gated  $Ca^{2+}$  channels (Yamada *et al.*, 1994).

Here we evaluate several cannabimimetic agents for their abilities to inhibit an aberrant pattern of glutamatergic synaptic activity and the neurotoxicity that results from it. Cannabinoid receptor agonists, acting via CB1 receptors, act potently and stereoselectively to inhibit  $[Ca^{2+}]_i$  spiking and neuronal death evoked by excitation of the synaptic network that forms in cultures of rat hippocampal neurons.

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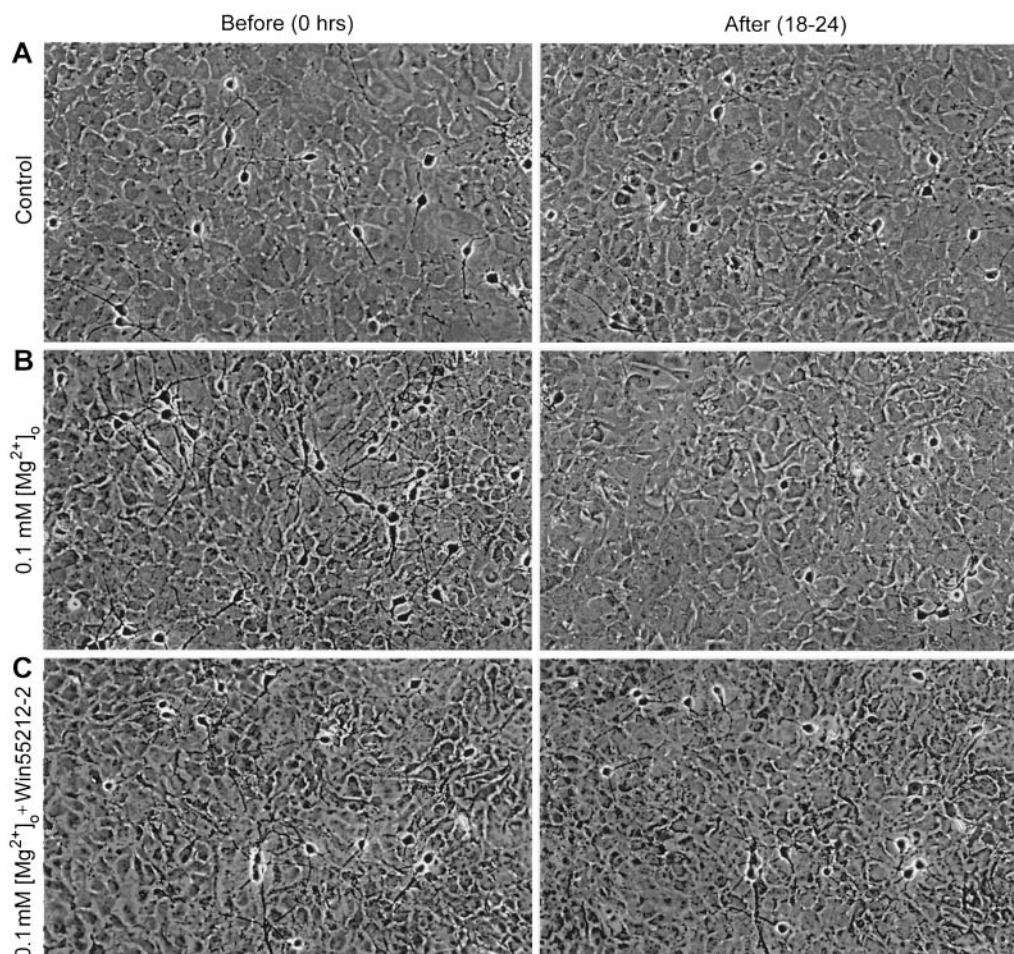
**ABBREVIATIONS:**  $[Ca^{2+}]_i$ , intracellular  $Ca^{2+}$  concentration;  $[Mg^{2+}]_o$ , extracellular  $Mg^{2+}$  concentration; NMDA, *N*-methyl-D-aspartate; CGS19755, ( $\pm$ )-2-amino-5-phosphonopentanoic acid; Win55212-2 (*R*-enantiomer), (+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-(1-naphthalenyl)methanone monomethanesulfonate; Win55212-3, *S*-enantiomer of Win55212; SR141716, *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide; CP55940,  $[1\alpha,2\beta(R),5\alpha]$ -(-)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenol.



**Fig. 1.** Cannabimimetics inhibit glutamatergic synaptic activity via CB1 receptors. Excitatory synaptic activity was induced by reducing  $[Mg^{2+}]_o$  to 0.1 mM, and the resulting  $[Ca^{2+}]_i$  spiking activity was recorded from a single hippocampal neuron using indo-1-based microfluorimetry. The  $[Mg^{2+}]_o$  was 0.1 mM throughout the recording, and 100 nM Win55212-2 and 100 nM SR141716 were applied by superfusion at the times indicated (horizontal bars).

## Materials and Methods

Materials were obtained from the following companies: NMDA, Win55212-2, Win55212-3, and CP55940 (levorotatory enantiomer), RBI (Natick, MA); SR141716, Sanofi Recherche (Montpellier, France); all other reagents, Sigma Chemical Co. (St. Louis, MO). Rat hippocampal neurons were grown in primary culture as previously described (Shen *et al.*, 1996). Neurons dissociated from hippocampi of embryonic day 17 rats were plated as droplets on microetched coverslips (Belco Biotechnology, Vineland, NJ), at a density of  $5 \times 10^4$  cells/well (approximately  $2.2 \times 10^4$  cells/cm<sup>2</sup>). Cultures were grown without mitotic inhibitors for a minimum of 12 days before use. For neurotoxicity experiments, at least 100 neurons were counted on each coverslip. Coverslips were then treated with the appropriate control or reduced- $[Mg^{2+}]_o$  solutions, and the same fields of cells were recounted 18–24 hr later. Drugs, when included, were added to the 0.1 mM  $Mg^{2+}$  medium before application to the cells. Viable neurons were identified based on morphological criteria; they were phase-bright, had rounded somata, and extended long fine processes. In some experiments, viability was confirmed by demonstrating that cells identified as viable also excluded propidium iodide (2  $\mu$ g/ml). We found that pairing pretreatment and post-treatment cell counts provided more reproducible assessments of the relatively modest degree of cell death resulting from this treatment. Analysis of variance with Bonferoni's *post hoc* test was used to determine significance. Data are presented as mean  $\pm$  standard error.  $[Ca^{2+}]_i$  was



**Fig. 2.** A cannabinoid receptor agonist protects cells against 0.1 mM  $[Mg^{2+}]_o$ -induced neurotoxicity. Phase-contrast photomicrographs show the same field of cultured rat hippocampal neurons before treatment (*left*) and 18–24 hr after treatment (*right*). Neurons (identified by a light halo around the soma and long fine processes) grew on a bed of non-neuronal cells that formed a mosaic beneath them. The neurons were treated with fresh medium (A), 0.1 mM  $[Mg^{2+}]_o$  (B), or 0.1 mM  $[Mg^{2+}]_o$  plus 100 nM Win55212-2 (C). Image contrast was enhanced digitally.

measured in single hippocampal neurons using indo-1-based microfluorimetry, as described previously (Shen *et al.*, 1996).

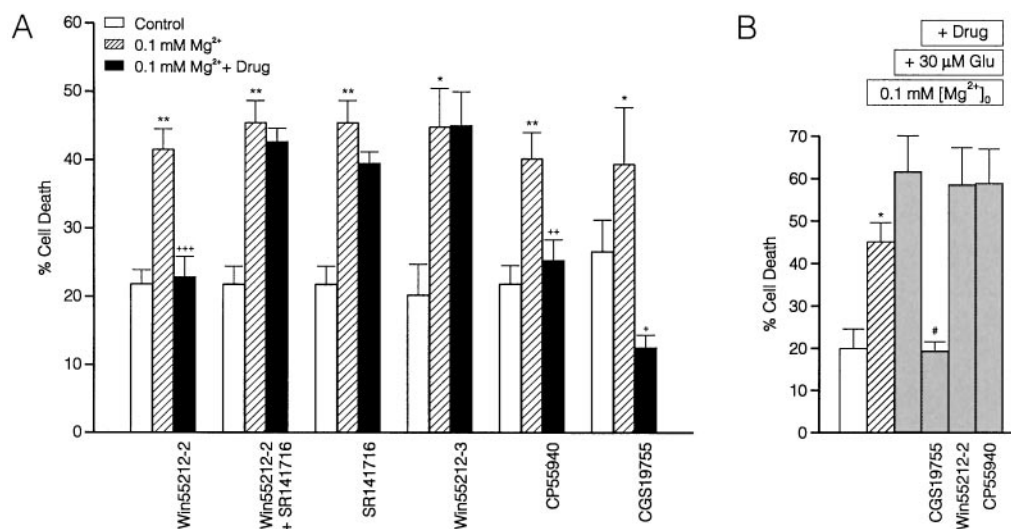
## Results

Reduction of  $[Mg^{2+}]_o$  to 0.1 mM elicits an intense pattern of  $[Ca^{2+}]_i$  spiking activity that depends on glutamatergic synaptic transmission (Shen *et al.*, 1996). As shown in Fig. 1, the cannabinoid receptor agonist Win55212–2 was a potent inhibitor of this synaptic activity. The Win55212–2-induced inhibition of low- $[Mg^{2+}]_o$ -induced  $[Ca^{2+}]_i$  spiking was mediated by the CB1 receptor, as indicated by the complete reversal of the effect by the CB1 receptor antagonist SR141716 (100 nM) (three experiments).

The excitatory synaptic activity elicited by reduction of the  $[Mg^{2+}]_o$  in central nervous system cultures has been shown to produce neurotoxicity, when sustained (Abele *et al.*, 1990; Rose *et al.*, 1990). The efficacy of cannabinimimetics in reducing  $[Ca^{2+}]_i$  spiking activity led us to hypothesize that cannabinoid receptor agonists would protect cells from this form of excitotoxicity. Cell viability was determined by counting the number of viable neurons before and 18–24 hr after treatment, as described in Materials and Methods. In control cultures (medium exchange only),  $22 \pm 2\%$  of the neurons died (Fig. 2A). This value is in good agreement with the previous observation that, as central nervous system cultures mature *in vitro*, increased synaptic activity parallels spontaneous cell death (Peterson *et al.*, 1989). Reducing  $[Mg^{2+}]_o$  to 0.1 mM increased neuronal death to  $42 \pm 3\%$  (19 experiments) (Fig. 2B), which was a significant increase relative to control cultures from the same plating ( $p < 0.001$ ). These levels of spontaneous and low- $[Mg^{2+}]_o$ -induced cell death are in good agreement with findings from previous reports describing the use of this model of synaptically mediated cell death (Abele *et al.*, 1990; Rose *et al.*, 1990). As shown in Figs. 2C and 3, 100 nM Win55212–2 was a very

effective neuroprotective agent. In the presence of Win55212–2, only  $23 \pm 3\%$  of the neurons died; this value is comparable to that seen in the control wells ( $22 \pm 2\%$ ) and is significantly less than that seen with 0.1 mM  $[Mg^{2+}]_o$  ( $p < 0.001$ ). The neuroprotection afforded by 100 nM Win55212–2 was blocked by 100 nM SR141716, consistent with a CB1-mediated process (Fig. 3A). The antagonist alone had no effect on cell viability. The inactive enantiomer Win55212–3 was also without effect. We previously showed that CP55940 acts as a partial agonist to inhibit low- $[Mg^{2+}]_o$ -induced  $[Ca^{2+}]_i$  spiking activity in these hippocampal cultures (Shen *et al.*, 1996). This compound was highly effective in preventing low- $[Mg^{2+}]_o$ -induced neurotoxicity, as shown in Fig. 3. Consistent with the glutamatergic mechanism of this excitotoxic model, the NMDA receptor antagonist CGS19755 (10  $\mu M$ ) not only prevented low- $[Mg^{2+}]_o$ -induced cell death but also improved viability, relative to the control cultures.

In a previous study, we found that CP55940 and Win55212–2 inhibited glutamatergic synaptic transmission, in identically prepared hippocampal cultures, by acting presynaptically to inhibit the release of glutamate (Shen *et al.*, 1996). If the neuroprotection we describe here resulted from inhibition of glutamate release, then cannabinimimetics would not be predicted to protect cells from the direct application of glutamate. This was indeed the case, as shown in Fig. 3B. Treatment of the cultures with 30  $\mu M$  glutamate in medium with 0.1 mM  $[Mg^{2+}]_o$  resulted in  $62 \pm 8\%$  cell death after a 18–24-hr exposure. This toxicity was completely prevented by including the NMDA receptor antagonist CGS19755 (10  $\mu M$ ) in the medium. However, neither Win55212–2 (100 nM) nor CP55940 (100 nM) influenced the survival of the cultures. Thus, cannabinimimetics protect cells from synaptically mediated excitotoxicity but not from exposure to exogenous glutamate.



**Fig. 3.** Cannabinimimetics are neuroprotective. Excitotoxicity was elicited by treatment with 0.1 mM  $[Mg^{2+}]_o$  (Fig. 2) and was quantified as described in Materials and Methods. The limited size of each culture plating precluded the testing of all treatments on a single plating. Therefore, each treatment has separate bars for control ( $\square$ ) and for 0.1 mM  $[Mg^{2+}]_o$ -induced death ( $\text{hatched}$ ). In all experiments, 0.1 mM  $[Mg^{2+}]_o$  induced significantly greater cell death, relative to control wells. Drugs were applied at the following concentrations: Win55212–2, 100 nM; SR141716, 100 nM; Win55212–3, 100 nM; CP55940, 100 nM; CGS19755, 10  $\mu M$ . A, Drugs were applied with 0.1 mM  $[Mg^{2+}]_o$  ( $\blacksquare$ ), as indicated below the histogram. B, Cells were treated for 18–24 hr with 0.1 mM  $[Mg^{2+}]_o$ , 30  $\mu M$  glutamate (Glu), and drug, as indicated (horizontal bars) above the histogram. The specific drugs added are indicated below the histogram. \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ , 0.1 mM  $[Mg^{2+}]_o$  versus control; +,  $p < 0.05$ ; ++,  $p < 0.01$ ; +++,  $p < 0.001$ , drug plus 0.1 mM  $[Mg^{2+}]_o$  versus 0.1 mM  $[Mg^{2+}]_o$ ; #,  $p < 0.001$ , glutamate plus 0.1 mM  $[Mg^{2+}]_o$  plus CGS19755 versus glutamate plus 0.1 mM  $[Mg^{2+}]_o$  (analysis of variance with Bonferroni's *post hoc* test).

## Discussion

In this report, we show that cannabinoid receptor agonists inhibit the  $[Ca^{2+}]_i$  spiking and neurotoxicity induced in hippocampal cultures by the reduction of  $[Mg^{2+}]_o$  to 0.1 mM. This action was stereoselective and mediated by the CB1 receptor. Excitotoxicity, such as that produced in this model system, is thought to underlie a number of neurodegenerative disorders, suggesting that cannabimimetics may prove useful in slowing neurodegenerative processes.

Cannabimimetic compounds with neuroprotective effects have been described previously, although this is the first description of neuroprotective effects mediated by the CB1 receptor. HU-211 was shown to protect neurons from NMDA-mediated neurotoxicity by acting as an antagonist at the NMDA receptor (Nadler *et al.*, 1993). The endogenous lipid palmitoylethanolamide, acting on CB2 receptors via an unknown mechanism, protected cultured cerebellar granule cells from the direct application of NMDA (Skaper *et al.*, 1996). In this report, we show that cannabinoids, acting through presynaptic CB1 receptors, protected cells from excitotoxicity. Cannabimimetics protected cells from the synaptic release of glutamate but not from the direct application of glutamate.

Excitotoxicity mediated by glutamate contributes to neuronal loss after acute insults, such as hypoxia-hypoglycemia, seizures, and head trauma (Meldrum, 1993; Choi, 1994; Rothman and Olney, 1995). A sensitization to glutamatergic input may also underlie more chronic neurodegenerative processes, such as Huntington's disease. Indeed, Beal *et al.* (1993) hypothesized that reduced block of the NMDA receptor by  $Mg^{2+}$  may underlie a number of neurodegenerative processes, suggesting that the *in vitro* model used here might identify drugs of use in these diseases. Furthermore, reductions in  $[Mg^{2+}]_o$  have been widely used to evoke epileptic discharges from a variety of neuronal preparations (Kohr and Heinemann, 1989; Abele *et al.*, 1990; Rose *et al.*, 1990). Our observation that cannabimimetics inhibit paroxysmal neuronal firing is consistent with previous reports indicating that cannabimimetics have antiepileptic properties (Yoshida *et al.*, 1995).

The neuroprotective efficacy of the compound CP55940 was of particular interest. This drug is a partial agonist that attenuates but does not block excitatory neurotransmission (Shen *et al.*, 1996). Drugs that completely block glutamatergic synaptic transmission have serious psychotomimetic side effects (Olney, 1989). In contrast, drugs that are partial agonists may prove to have a greater margin of safety. Indeed,  $\Delta^9$ -tetrahydrocannabinol acts as a partial agonist to inhibit excitatory neurotransmission (Shen and Thayer, 1997), consistent with the safety (at least in terms of acute lethality) of drugs that attenuate but do not block glutamatergic synaptic transmission.

In conclusion, our data demonstrate that cannabinoid receptor agonists prevent excitotoxicity *in vitro*. We hypothesize that cannabimimetics would reduce the size of lesions in animal models of stroke, epilepsy, and metabolic disease, and we speculate that these drugs might be useful in treating neurodegenerative disorders.

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