

CANNABINOIDS ALTER NEUROTOXICITY PRODUCED BY INTERLEUKIN-6 IN CENTRAL NERVOUS SYSTEM NEURONS

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1. INTRODUCTION

Abnormal levels of cytokines, including interleukin-6 (IL-6), are found in the cerebrospinal fluid and brain of individuals infected with the human immunodeficiency virus (HIV)^{6,16,19} and may contribute to the central nervous system (CNS) pathology and cognitive dysfunction observed in individuals with **NeuroAIDS**. Consistent with this possibility, transgenic mice that overexpress IL-6 (IL-6 transgenic mice) in the CNS show CNS pathology that resembles the neuropathology observed in HIV-infected individuals". Moreover, electrophysiological investigations of the IL-6 transgenic mice indicate that the functional properties of CNS **neurons**¹³ and **neuronal circuits**^{3,17} are altered by IL-6 overexpression in the CNS, and behavioral studies show that cognitive deficits occur as well⁸.

The mechanisms mediating **IL-6's** effects on the CNS are still under investigation. Both CNS neurons and **glial** cells express IL-6 receptors and are potential contributors to **IL-6's** actions in the CNS. IL-6 receptors are linked to an intracellular signal transduction subunit referred to as **gp130**, which has been shown to initiate **tyrosine** kinase activity and gene expression in the non-neuronal cell types where it has been studied'. Receptors for growth factors such as leukemia inhibitory factor (LIF) and **ciliary** neurotrophic factor (CNTF) also utilize the **gp130** transduction pathway', suggesting that this pathway may play an important role in **neuronal** development and plasticity. Thus, developing neurons may be an advantageous model to identify intracellular pathways and **neuronal** functions

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affected by IL-6 exposure. Recently we showed in primary cultures of rat **cerebellar** granule neurons, a **well** characterized *in vitro* developmental model system, that chronic IL-6 treatment of the granule neurons during development increased resting Ca^{2+} levels and the Ca^{2+} signal to N-methyl-D-aspartate (NMDA), an agonist at the NMDA subtype of glutamate receptor⁶. The granule neurons are excitatory neurons that use glutamate as a transmitter and receive synaptic input mediated by glutamate. Immunostaining showed that the granule neurons express IL-6 receptors and the intracellular signal transduction subunit gp 130⁵.

The effect of IL-6 on resting Ca^{2+} levels and NMDA receptor (NMDAR)-mediated Ca^{2+} signals could have a significant impact on other granule neuron functions. Intracellular Ca^{2+} is an important second messenger and regulates numerous **neuronal functions** such as transmitter release, gene expression and membrane excitability. **Neurotransmission** via glutamate receptors (**GluRs**), and in particular NMDARs, is a major pathway for Ca^{2+} entry into CNS neurons. However, excessive activation of NMDARs can lead to Ca^{2+} overload and neurotoxicity, and this pathway is thought to contribute to the neuropathology of **NeuroAIDS**¹⁰. Thus, **IL-6's** effects on NMDAR-induced changes in intracellular Ca^{2+} may be a contributing factor in neuropathological changes associated with conditions of elevated IL-6 in the CNS such as occurs in NeuroAIDS.

The disruptive influence of HIV infection in the **CNS** is likely to be exacerbated when combined with other factors that cause altered **neuronal** function such as drugs of abuse. To pursue this possibility at the cellular level, we have been examining the effects of **cannabinoids** on cultured granule neurons under control conditions and after chronic treatment of the cultured granule neurons with IL-6 to simulate conditions that occur in HIV infection. Cannabinoids are the active ingredients in marijuana and hashish, drugs that are abused commonly in the HIV-infected and non-infected populations. The behavioral actions of cannabinoids are well known¹¹; however, effects of cannabinoids at the cellular level have yet to be fully elucidated, and few studies have pursued potential interactions between cannabinoid and factors such as IL-6 that have been proposed to contribute to the neuropathology of NeuroAIDS. CNS neurons appear to express only one type of cannabinoid receptor (CbR1) and granule neurons are known to express an abundance of this **receptor**⁴. Thus, granule neurons are a favorable model to study the physiological and pathological actions of cannabinoids as well as interactions between cannabinoids and IL-6.

In initial studies we found that acute exposure of control cultured granule neurons to CbR1 agonists elevates resting intracellular Ca^{2+} levels and enhances the Ca^{2+} signal to NMDA, effects similar to that observed with chronic IL-6 treatment of the granule **neurons**⁷. These results raise the possibility that cannabinoids could produce neurotoxicity through NMDAR and increased intracellular Ca^{2+} levels. Moreover, cannabinoid effects on intracellular Ca^{2+} levels and the Ca^{2+} signal to NMDA could interact with the effects of IL-6 in neurons exposed to both IL-6 and cannabinoids, increasing the potential for **neurotoxicity**. Such an interaction could have important implications for HIV-infected drug users. To address these issues, in the current studies we have assessed the ability of cannabinoids and IL-6 to induce neurotoxicity in granule neuron cultures when applied alone or together. For comparison, we have also assessed the ability of cannabinoids to alter NMDA-induced toxicity. Neurotoxicity was assessed by two standard assays: (a) measurement of lactate dehydrogenase (LDH) levels in the culture supernatant, and (b) measurement of mitochondrial enzyme function in the granule cell layer. LDH is a **cytoplasmic** enzyme and its presence in cell supernatants is indicative of cell damage. **Mitochondrial** enzyme function is used commonly to assess cell viability. Results indicate that IL-6 treatment induces cell damage and decreases **neuronal** viability, and that under some conditions cannabinoids alter these effects.

2. MATERIALS AND METHODS

2.1. Tissue Culture

Granule neuron cultures were prepared from cerebella of postnatal Sprague-Dawley rats according to a standard enzyme dissociation method published previously¹⁴. Briefly, cerebella were removed from the brains of 8-day-old pups and granule neurons isolated by dissociating with trypsin and centrifugation to collect the cells. The granule neurons were plated on MATRIGEL® (Collaborative Biomedical Products, Bedford, MA) coated glass coverslips at a density of 4.0×10^6 cell/ml in growth medium consisting of DMEM/F12 supplemented with 10% horse serum, 30 mM glucose, 2 mM glutamine, 25 mM KCl and 25 μ M penicillin-streptomycin. The cultures were maintained in a standard CO₂ incubator at 37°C. 5-fluorodeoxyuridine (FUDR; 20 μ g/ml) was added to the media on the 1st and 4th days after plating to reduce the number of non-neuronal cells in culture. The cellular composition of the cultures was determined by immunostaining with antibodies specific for proteins localized to various cerebellar cell types (e.g., glial fibrillary acidic protein for astrocytes, GABA for inhibitory interneurons; glutamate for granule neurons) and indicated that greater than 95% of the cells in culture were granule neurons.

2.2. IL-6 Treatment

Human recombinant IL-6 (Boehringer Mannheim, Germany) was dissolved in physiological saline at 100,000 units/ml as stock solution and stored at -20°C. IL-6 at a concentration of 10 ng/ml (1000 units/ml) was used in most studies and was added to the granule neuron cultures at 1 and 4 days in vitro (DIV). In some experiments lower concentrations of IL-6 or heat denatured (100°C, 1 hr) IL-6 were used. Control cultures (sister cultures) were untreated with IL-6.

2.3. Neurotoxicity

Experiments to measure cell damage and neuronal viability were started at 7 DIV and completed at 8 DIV. Exposure to IL-6 was terminated at the start of the assays (7 DIV). The same cultures were used to assess both neuronal viability via the MTT assay and cell damage via the LDH assay. LDH levels were determined in the culture supernatant, whereas the cell layer was used for the neuronal viability assay. Measurements were made in control, cannabinoid-treated, IL-6-treated, and IL-6 plus cannabinoid-treated cultures. In some studies NMDA-treated and NMDA plus cannabinoid-treated cultures were examined as well.

To assess the ability of CbR1 agonists and NMDA to alter neuronal viability and induce cell damage, we used a paradigm commonly used to measure NMDA-induced toxicity. This paradigm assessed toxicity that occurs during a 24 hr period following an initial insult. In the current experiments, the initial insult was a 1 hr exposure to NMDA and/or cannabinoids. All cultures were treated with this protocol except that control and some of the IL-6 treated cultures were exposed to saline alone (instead of cannabinoid, NMDA, or cannabinoid/NMDA) during the acute treatment period. For the acute treatment, the culture medium was removed and replaced with Mg²⁺-free physiological saline consisting of (in mM): 140 NaCl, 3.5 KCl, 0.4 KH₂PO₄, 1.25 Na₂HPO₄, 2.2 CaCl₂, 10 glucose, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.3), 25 μ M penicillin-streptomycin, and 1% bovine serum albumin (BSA). Mg²⁺ was excluded from the

saline to prevent blockade of NMDAR by this ion. In addition, 5 μ M glycine was added to the saline since this amino acid is considered to be required as a co-agonist for NMDAR activation. After the 1 hr incubation period, the cultures were rinsed with saline and serum-free growth medium containing 1% BSA was added. The cultures were then incubated (37°C) for an additional 24 hr after which time the culture supernatant was subjected to the LDH assay and the cell layer subjected to the MTT assay.

The LDH level in the supernatant of each culture was determined spectrophotometrically using a commercially available kit (Boehringer Mannheim). Absorbency values for drug-treated cultures were normalized to the mean value for control cultures from the same culture set. Cell viability was quantified by measuring mitochondrial activity using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT is a compound that is reduced to an insoluble salt by succinate dehydrogenase, an enzyme located in the mitochondria of cells. The extent of this reaction was determined spectrophotometrically. Absorbency values for drug-treated cultures were normalized to the mean absorbency value for control cultures from the same culture set and expressed as the percent viability relative to control cultures. For both LDH and MTT assays, normalized data from different culture sets were pooled and analyzed by ANOVA. $P \leq 0.05$ was considered indicative of a significant difference; $0.10 > P > 0.05$ was considered a trend in the data.

3. RESULTS

The granule neurons were treated chronically with IL-6 during the main period of morphological development in culture, from 1 DIV to 7 DIV. Figure 1 shows the morphological features of the granule neuron cultures at 1 DIV (Fig. 1A) and at 7 DIV (Fig. 1B). The first day *in vitro* (1 DIV) represents an early stage in granule neuron development, when the neurons are extending processes and migrating to form neuronal clusters. By 7 DIV, the majority of granule neurons are located in neuronal clusters and express long processes that form fiber tracts that interconnect the neuronal clusters. Chronic treatment with IL-6 did not alter the general features of this process.

Chronic IL-6 treatment (1000 units/ml) of the granule neuron cultures for the 6 day period significantly reduced neuronal viability as measured by the MTT assay. This effect

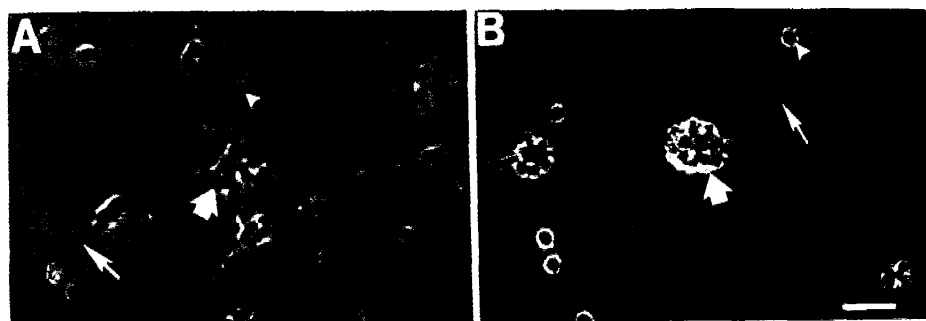


Figure 1. Phase contrast micrographs showing morphological features of granule neurons at two culture ages, 1 DIV (A) and 7 DIV (B). When plated, the granule neurons are rounded in shape. By 1 DIV they have attached to the substrate, extended processes, and started to migrate to form neuronal clusters. By 7 DIV prominent clusters are evident with long fiber tracts connecting the clusters. Large arrows point to neuronal clusters; small arrowheads point to single granule neurons; medium sized arrows point to processes (1 DIV) or fiber tracts (7 DIV). Calibration bar=40 μ m.

was not observed when the cultures were treated with denatured IL-6, indicating that the effect on neuronal viability was specific to the active form of IL-6 (Fig. 2A). Consistent with a toxic effect of IL-6, levels of the cytoplasmic enzyme LDH in the culture supernatants were significantly higher in IL-6 treated cultures compared to control cultures, indicating that IL-6 treatment produces cell damage (Fig. 2B).

Acute treatment (1 hr) with the cannabinoid receptor agonist R(+)-WIN 55,212-2 (R(+)-WIN) influenced neuronal viability in control and IL-6-treated cultures as determined by the MTT assay (measured 24 hr after cannabinoid exposure). Control cultures exposure to 10 nM R(+)-WIN, the lowest dose tested, showed a trend toward increase neuronal viability compared to untreated control cultures (Fig. 3A). In the IL-6-treated cultures, 10 nM R(+)-WIN significantly increased neuronal viability, reversing the negative effect of IL-6 treatment on neuronal viability (Fig. 3B). This effect of R(+)-WIN was not observed with S(-)-WIN, less active enantiomer, suggesting that CbR1s were involved in this action of R(+)-WIN. At higher concentrations, neither R(+)-WIN or S(-)-WIN significantly affected neuronal viability in the control or IL-6 treated cultures (Fig. 3).

Acute treatment (1 hr) of control and IL-6-treated granule neuron cultures with R(+)-WIN also influenced cell damage as indicated by LDH levels in the culture supernatant (measured 24 hr after cannabinoid exposure). In control cultures, 10 nM R(+)-WIN had no significant effect on baseline LDH levels (Fig. 4A). However, higher concentrations of R(+)-WIN significantly reduced LDH levels compared to untreated control cultures, an effect that was also observed for S(-)-WIN (Fig. 4A). Similar results were observed in the IL-6-treated cultures (Fig. 4B). At 10 nM neither R(+)-WIN or S(-)-WIN affected the IL-6-induced elevation in LDH levels. However at higher doses, both R(+)-WIN and S(-)-WIN reduced the IL-6-induced elevation in LDH levels.

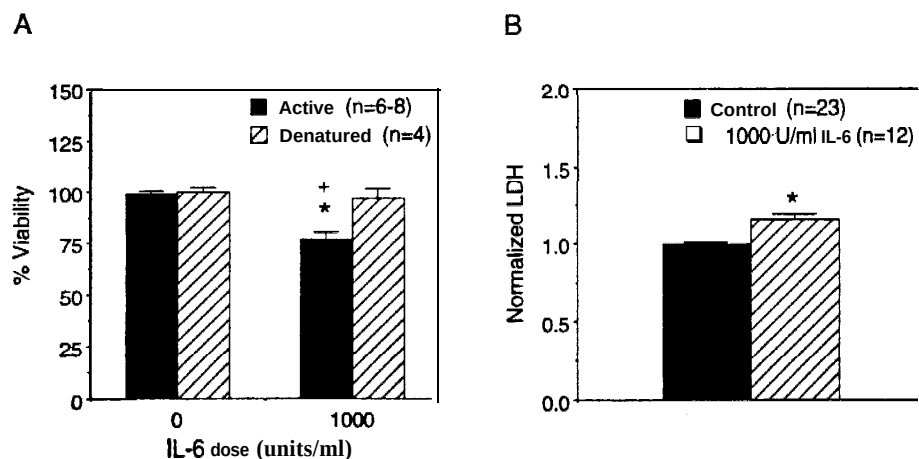


Figure 2. Chronic IL-6 treatment induces neurotoxicity in cerebellar granule neuron cultures. **A.** Mean ($\pm$ S.E.M.) values for the % viability in control and IL-6-treated cultures. Data are normalized to the mean value for control cultures from the same culture set. A decrease in cell viability (MTT assay) was observed in the IL-6-treated cultures compared to untreated control cultures. A similar concentration of heat-denatured IL-6 did not produce the same decrease in viability as active IL-6, indicating that the change in cell viability was specific to the active form of IL-6. **B.** Mean ($\pm$ S.E.M.) values for LDH levels in the culture supernatant of control and IL-6-treated cultures. LDH levels were normalized to the mean LDH level in control cultures from the same culture set. LDH levels were elevated in the culture supernatant of IL-6 treated cultures compared to control cultures indicating a toxic effect of the IL-6 treatment. * = significant difference from baseline control; • = significant difference from denatured IL-6 ($p < 0.05$, ANOVA). n = number of cultures measured.

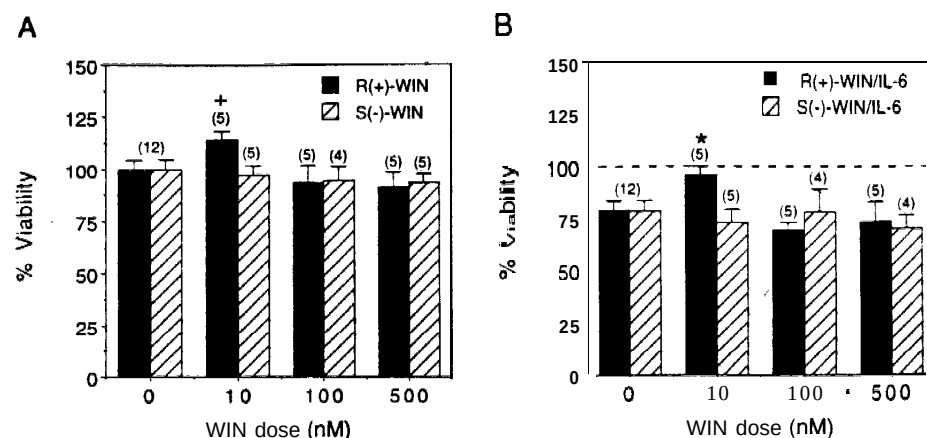


Figure 3. Effect of acute treatment with the cannabinoid receptor agonist R(+)-WIN and the less active isomer S(-)-WIN on neuronal viability in control and IL-6 treated cultures. **A,B.** Mean $\pm$ SEM values for % viability in control (A) and IL-6-treated (B) cultures. Data are normalized to the mean value for control cultures from the same culture set. Acute treatment with R(+)-WIN at a concentration of 10 nM significantly enhanced neuronal viability in both control and IL-6-treated cultures. S(-)-WIN did not produce a similar effect. At higher concentrations, neither R(+)-WIN or S(-)-WIN affected neuronal viability in control or IL-6-treated cultures. * = significant difference ($P \leq 0.05$, ANOVA) from baseline levels (i.e., at WIN concentration = 0). * = a trend in the data ($0.10 > P > 0.05$, ANOVA). Numbers in parenthesis are the number of cultures measured.

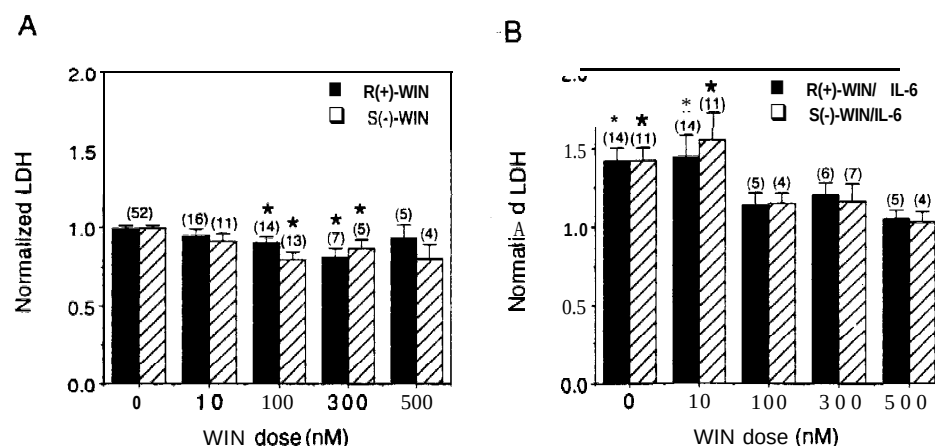


Figure 4. Induction of cell damage by IL-6 and cannabinoid receptor agonists. **A.** Mean $\pm$ SEM values for normalized LDH levels in the culture supernatant of control neurons treated acutely with R(+)-WIN or S(-)-WIN. Data are normalized to the mean value for control cultures from the same culture set. Both WIN compounds reduced LDH levels. **B.** Mean $\pm$ SEM values for normalized LDH levels in the culture supernatant of neurons exposed to IL-6 (1000 U/ml) for 6 days and then treated acutely with R(+)-WIN or S(-)-WIN. IL-6 treatment significantly increased LDH levels compared to levels in control cultures. The effect of IL-6 was not altered by acute treatment with 10 nM R(+)-WIN or S(-)-WIN. At higher concentrations of R(+)-WIN or S(-)-WIN, LDH levels in IL-6 treated cultures were not significantly different from that observed in control cultures (i.e., no IL-6) indicating that the cannabinoids reduced the cell damage due to IL-6. * = significant difference ($p \leq 0.05$, ANOVA) from control (no IL-6). Numbers in parenthesis are the number of cultures measured.

In additional experiments, we assessed the ability of 10 nM R(+)-WIN or S(-)-WIN to affect the cell damage produced by lower doses of IL-6. Cultures treated with IL-6 at 100 units/ml or 500 units/ml for 6 days were exposed to acute WIN for 1 hr and LDH levels assessed 24 hours later, as was done for the cultures treated with 1000 units/ml of IL-6. Although the cell damage was less with the lower concentrations of IL-6, neither R(+)-WIN nor S(-)-WIN consistently affected the level of damage as indicated by their lack of effect on LDH levels induced by the lower doses of IL-6 (Fig. 5).

Acute application of NMDA to granule neurons evokes an intracellular Ca^{2+} signal that contributes to NMDA-induced cell damage under conditions of excessive NMDA stimulation'. Acute application of R(+)-WIN enhances the Ca^{2+} signal to NMDA⁷. Thus it was of interest to determine if co-application of WIN with NMDA could enhance NMDA-induced cell damage. To assess this, two doses of NMDA, 10 μM and 500 μM , and two doses of R(+)-WIN, 20 nM and 100 nM, were tested. These concentrations of R(+)-WIN produce a large increase (50–100%) in the Ca^{2+} signal to NMDA, and thus would be expected to enhance NMDAR- and Ca^{2+} -linked cell damage. The same protocol was used as for cannabinoid treatment describe above (1 hr treatment; LDH levels measured -24 hours later). NMDA at 10 μM did not induce cell damage, and this lack of effect was not altered by co-exposure to R(+)-WIN or S(-)-WIN (Fig. 6). NMDA at 500 μM induced significant cell damage, however, neither R(+)-WIN nor S(-)-WIN (20 nM and 100 nM)

Figure 5. R(+)-WIN and S(-)-WIN at 10 nM did not alter neuronal damage produced by IL-6 at doses ranging from 100 units/ml to 1000 units/ml. Mean $\pm$ SEM values for LDH levels in the culture supernatant of control and IL-6 treated cultures exposed to acute WIN. Data are normalized to the mean value for control cultures from the same culture set. Chronic treatment with IL-6 induced a dose-dependent increase in LDH levels. These levels were not altered by R(+)-WIN and S(-)-WIN at 10 nM. * = significant difference ($p \leq 0.05$, ANOVA) from control (no IL-6). Numbers in parenthesis are the number of cultures measured.

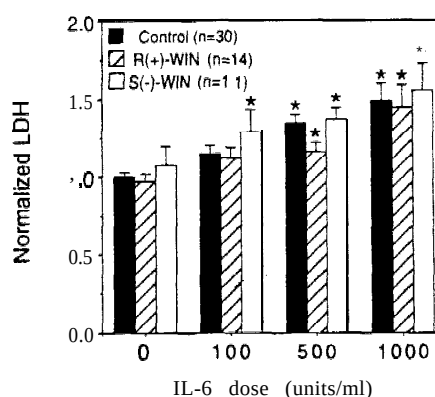
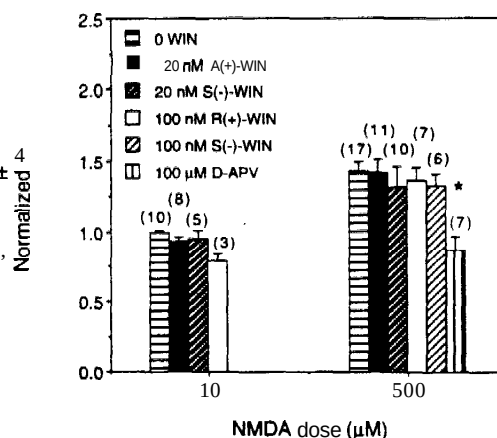


Figure 6. R(+)-WIN and S(-)-WIN at 10 nM did not alter NMDA-induced neuronal damage. Mean $\pm$ SEM values for normalized LDH levels (normalized to baseline control values in the absence of NMDA or WIN) in the culture supernatant of NMDA, NMDA plus R(+)-WIN or plus S(-)-WIN at 10 nM, or NMDA plus APV. NMDA at 10 μM did not induce neuronal damage as indicated by LDH levels, whereas at 500 nM significant neuronal damage was observed. * = significant difference ($p \leq 0.05$, ANOVA) from control. Numbers in parenthesis are the number of cultures measured.



influenced the NMDA-induced cell damage. NMDA-induced cell damage was blocked by the NMDA receptor antagonist ($\pm$)-2 amino-5 phosphonopentanoic acid (B-APV), indicating that the cell damage was dependent on NMDAR activation (Fig. 6).

4. DISCUSSION

Results from the current study show that chronic treatment of developing cultured granule neurons with the cytokine IL-6 at concentrations considered to be **pathophysiological** (1000 units/ml) produces cell damage and decreases **neuronal** viability. These results demonstrate that IL-6 can be neurotoxic and support a role for IL-6 in the neuropathology observed in diseases associated with elevated levels of IL-6 in the CNS such as **NeuroAIDS**.

The toxicity induced by chronic treatment with IL-6 was altered by acute treatment with the cannabinoid receptor agonist **R(+)-WIN**. A low dose (10 nM) of **R(+)-WIN** increased cell viability in the IL-6 treated cultures, effects not mimicked by the less active enantiomer **S(-)-WIN**. Similar results were observed in control cultures, suggesting that this effect of **R(+)-WIN** was not specific to IL-6 induced toxicity but also affected baseline toxicity. The cause of the baseline toxicity is unknown but may reflect programmed cell death that normally occurs as part of the developmental program in CNS neurons. Alternatively, baseline cell death could result from damage due to the experimental manipulations. Although **R(+)-WIN** at 10 nM enhanced **neuronal** viability in the IL-6 treated cultures, **neuronal** damage as indicated by LDH levels in the culture supernatant were not reduced. One possible explanation for this discrepancy is that cannabinoid treatment enables the cells to recover from damage and consequently results in increased viability.

At higher doses of **R(+)-WIN** (100–500 nM), acute treatment of both control and IL-6 treated cultures decreased cell damage (LDH assay). This effect did not appear to be mediated by **CbRs**, since both **R(+)-WIN** and **S(-)-WIN** were effective. The decrease in cell damage produced by acute treatment with **R(+)-WIN** and **S(-)-WIN** was not associated with an increase in **neuronal** viability as measured by the MTT assay, perhaps because changes in viability occur on a longer time scale than that utilized in the current study, or because the decreased cell damage was not sufficient to cause a measurable change in cell viability. Alternatively, the decreased cell damage reflects a switch from a necrotic cell death pathway to an apoptotic cell death pathway. Necrotic cell death is characterized by membrane breakdown and leakage of LDH out of the cell. In apoptotic cell death, cell membranes remain intact preventing LDH leakage and mitochondrial function continues until relatively late in the process. Thus, a decrease in LDH levels without an concomitant increase in viability could reflect a switch in the toxic pathway from necrotic to **apoptotic** rather than a protective process. Future studies will assess the possible involvement of an apoptotic pathway in the actions of IL-6 and cannabinoids in the granule neurons.

Both **R(+)-WIN** and **IL-6**¹⁴ increase resting Ca^{2+} levels, an effect that could lead to Ca^{2+} overload and toxicity. However, in the current studies IL-6 treatment produced cell damage as indicated by an increase in LDH levels in the culture supernatant, whereas **R(+)-WIN** did not increase LDH levels. Moreover, **R(+)-WIN** did not potentiate the cell damage induced by chronic treatment with IL-6 or the damage produced by acute treatment with NMDA, in spite of the fact that **R(+)-WIN** at low concentrations (e.g., 10 nM) increases (50–100%) the Ca^{2+} signal to NMDA.⁷ Taken together, these results suggest that although **R(+)-WIN** influences intracellular Ca^{2+} levels it has little toxic potential when applied at a low dose for a short duration and may in fact be beneficial, as indicated by the increased viability in the IL-6 treated cultures exposed to 10 nM **R(+)-WIN**. Studies are in

progress to determine the toxic potential of long duration applications of cannabinoids. The lack of dose-dependency for the effect of R(+)-WIN on neuronal viability may reflect the involvement of different intracellular pathways at different R(+)-WIN doses. CbR1 is known to be linked to an inhibition of adenylate cyclase via a G-protein⁴ and to influence neuronal function directly via the G-protein¹⁵ and via a change in intracellular cAMP levels⁴. The effects involving cAMP typically occur at higher doses⁴ than the direct G-protein action¹⁵. Thus, actions of R(+)-WIN at the higher doses (100-500 nM) could reflect a mixed effect of R(+)-WIN involving more than one intracellular pathway.

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