Published in final edited form as:

Neuropharmacology. 2010 February ; 58(2): 551–558. doi:10.1016/j.neuropharm.2009.07.035.

Cannabinoid Receptor Activation Reduces TNF α -Induced Surface Localization of AMPAR-Type Glutamate Receptors and Excitotoxicity

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Abstract

After injury or during neurodegenerative disease in the central nervous system (CNS), the concentration of tumor necrosis factor alpha (TNF α) rises above normal during the inflammatory response. *In vitro* and *in vivo*, addition of exogenous TNF α to neurons has been shown to induce rapid plasma membrane-delivery of AMPA-type glutamate receptors (AMPA-Rs) potentiating glutamatergic excitotoxicity. Thus the discovery of drug targets reducing excess TNF α -induced AMPAR surface expression may help protect neurons after injury. In this study, we investigate the neuroprotective role of the CB1 cannabinoid receptor using quantitative immunofluorescent and real-time video microscopy to measure the steady state plasma membrane AMPAR distribution and rate of AMPAR exocytosis after TNF α exposure in the presence or absence of CB1 agonists. The neuroprotective potential of CB1 activation with TNF α was measured in hippocampal neuron cultures challenged by an *in vitro* kainate (KA)-mediated model of Excitotoxic Neuroinflammatory Death (END). Here, we demonstrate that CB1 activation blocks the TNF α -induced increase in surface AMPARs and protects neurons from END. Thus, neuroprotective strategies which increase CB1 activity may help to reduce the END that occurs as a result of a majority of CNS insults.

Keywords

AMPA receptor; cannabinoid receptor; excitotoxicity; glutamate receptor; neuroprotection

1. Introduction

Control over normal neuronal glutamatergic signaling depends largely on the localization of AMPARs on synaptic and extrasynaptic plasma membrane (Malinow and Malenka, 2002). This balance can be disrupted during CNS injury or disease when the inflammatory response is initiated and the population of AMPARs on neuronal plasma membrane increases dramatically, potentiating END (reviewed in (Leonoudakis et al., 2004; Pickering et al.,

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2005). We previously characterized TNF α as a powerful glial-derived instigator of AMPAR trafficking to the plasma membrane (Beattie et al., 2002; Stellwagen et al., 2005). The presence of TNF α in the CNS is required for synaptic development (Stellwagen and Malenka, 2006), but the increased TNF α concentrations induced following infection or injury (Nemeth et al., 1997) contribute to END (Lock et al., 1999; Nagatsu et al., 2000; New et al., 1998; Perry et al., 2001; Shohami et al., 1999; Szelenyi, 2001). Several groups attribute this toxicity to long-term, translation-dependent apoptotic signaling pathways (Fontaine et al., 2002; Reimann-Philipp et al., 2001; Yang et al., 2002; Zhao et al., 2001; Zou and Crews, 2005). However, TNF α also induces a rapid (within 15 minutes) increase in surface expression of AMPARs (Beattie et al., 2002; Ogoshi et al., 2005; Stellwagen et al., 2005) which are calcium permeable and principally localized extrasynaptically (Leonoudakis et al., 2008). Additionally, acute neurotrauma increases surface expression of calcium permeable AMPARs composed of GluR1 subunits (Grooms et al., 2000; Grossman et al., 1999; Sanchez et al., 2001; Ying et al., 1998), and potentiates neuronal excitotoxicity (Feldmeyer et al., 1999; Oguro et al., 1999). Together, these studies suggest that TNF α -induced AMPAR surface expression may be a mechanism contributing to the early stages of END. Agents which decrease TNF α -induced AMPAR surface expression may reduce excitotoxicity in neurons and our previous studies suggest the use of cannabinoid receptor agonists may serve as a potential pharmacological intervention (Abood et al., 2001).

The cannabinoid receptor system is found in hippocampal neurons (Leterrier et al., 2006; Mackie, 2007; McDonald et al., 2007) and protects against excitotoxicity (Kim et al., 2006; Marsicano et al., 2003; Shen and Thayer, 1998, 1999). Activation of the G-protein coupled CB1 cannabinoid receptor initiates complex signaling cascades and leads to the hyperpolarization of neuronal membranes by increasing potassium and decreasing calcium conductance (Howlett, 2002). Our current results suggest a novel neuroprotective mechanism under the control of CB1, namely the reduction of excessive AMPAR surface expression caused by TNF α . We have used hippocampal culture systems to examine AMPAR surface expression and neuron survival after KA-induced excitotoxicity while exogenously applying TNF α . Our results demonstrate that CB1 activation prevents the TNF α -induced increase in surface GluR1-containing AMPARs and protects neurons from TNF α potentiated excitotoxic stress.

2. Materials and Methods

2.1. Preparation of hippocampal cultures

Mixed hippocampal neuron cultures were prepared from E18 rat pups as previously described (Beattie et al., 2002). Briefly, hippocampi of embryonic day 18 (E18) Sprague Dawley rats were removed, digested with papain, and dissociated by trituration. Neurons were plated in Neurobasal medium containing B27 supplement and Glutamax (Invitrogen) on coverslips or plastic plates coated with poly-D-lysine. After cell attachment (3–4 h), the medium was replaced. Three days later, an equal volume of Neurobasal medium containing N2 supplement and Glutamax was added. Cultures were fed weekly by replacing one-half of the medium with Neurobasal/N2/Glutamax. Astrocyte growth was inhibited by adding 5-fluoro-2'-deoxyuridine after 6 days *in vitro* (div). Cultures were used for experiments between weeks 3 and 4.

2.2. Immunofluorescence AMPAR surface localization

Neurons were fixed and processed as described (Leonoudakis et al., 2008). Before neuron treatment, the medium was changed to prewarmed artificial CSF (ACSF) (25 mM HEPES, pH 7.4, 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 33 mM D-glucose with the following receptor antagonists: 0.5 μ M tetrodotoxin, 3 μ M strychnine, 20 μ M

bicuculline methiodide, and 20 μ M D-APV to eliminate the contributions of spontaneous action potentials, inhibitory signaling, and NMDA receptor activation, respectively) for 30 minutes. The cannabinoid receptor agonists WIN55212-2 (0.5 μ M, Sigma, St. Louis, MO), THC (0.5 μ M, RTI), 2AG (0.5 μ M, Tocris), and anandamide (0.5 μ M, Tocris) were added 30 min before TNF α treatment. Recombinant rat TNF α (6 nM, R&D Systems) was then added to cells for an additional 15 minutes. Cannabinoid receptor antagonists (SR141716A, SR144528 and VCHSR1-used at 0.5 μ M) were added 5 min before agonist application. Antagonists were provided by Drs. Herb Seltzman (RTI, Research Triangle Park, NC) and Patricia Reggio (UNC Greensboro). After the indicated times, neurons were fixed with 4% paraformaldehyde/4% sucrose in PBS for 10 min, washed with PBS, blocked with 3% BSA, 2% goat serum in PBS (blocking buffer) for 1 h, and incubated with rabbit anti-GluR1 (Calbiochem) or rabbit anti-CB1 (Sigma-Aldrich) antibodies (both against extracellular epitopes) and with goat anti-rabbit Alexafluor 555 and/or goat anti-mouse Alexafluor 488 (Invitrogen). For surface CB1 colocalization with synaptic markers, cells were permeabilized with 0.2% Triton X-100 and incubated with mouse monoclonal anti-PSD-95 (Affinity BioReagents, Inc.) or synaptophysin (Sigma-Aldrich) antibodies. Colocalization of PSD-95 or synaptophysin with CB1 was examined by imaging neurons with a single 0.5 μ m thick plane at 1024 $\times$ 1024 pixel resolution with a Nikon C1 confocal microscope. Steady-state surface expression of AMPARs was determined as described by reports from our lab and others (Beattie et al., 2002; Gao et al., 2006; Leonoudakis et al., 2008; Ogoshi et al., 2005; Stellwagen et al., 2005). For individual experiments, images for all conditions were analyzed using identical acquisition parameters and untreated and treated cells from the same culture preparation were compared. Regions of equal size containing dendrites 30 μ m away from the cell body were used for quantification. AMPAR surface expression level is represented as a ratio of surface AMPAR area divided by the total dendritic area in the visual field using MetaMorph software (Molecular Devices). Each experiment was repeated at least three times, and individual normalized cell values were averaged for each experimental condition.

2.3. Live imaging of superecliptic pHluorin-tagged GluR1 in hippocampal neurons

AMPA exocytosis events were visualized by transfection of hippocampal neurons by AMAXA nucleofection with cDNA encoding superecliptic pHluorin (SEP)-tagged GluR1 imaged with live video microscopy as described except for the composition of the imaging media (Yudowski et al., 2007). Live neurons were imaged in 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 10 mM D-glucose, 25 mM HEPES, pH 7.4., 0.5 μ M tetrodotoxin, 20 μ M bicuculline methiodide, 20 μ M CNQX, and 50 μ M D-APV in the presence or absence of WIN (0.5 μ M), TNF α (100ng/ml), or WIN+TNF α . Temperature of the imaging chamber was maintained at 37°C. The rate of AMPAR exocytosis events was measured in real time (100 msec time resolution). Sixty second videos of AMPAR exocytosis were collected from dendritic fields of over 100 separate hippocampal neurons exposed to four different conditions. For exocytotic rate analysis after WIN or WIN+TNF α treatment, neurons were pretreated with WIN for at least 15 minutes prior to examination. Then during WIN+TNF α treatment neurons were examined within a 15–45 minute window after this TNF α application which we have determined is the maximum AMPAR surface delivery time zone after TNF α application (Leonoudakis et al., 2008). Standard criteria were used to ensure that only healthy pyramidal hippocampal neurons transfected with comparable amounts of SEP-GluR1 were used for analysis (Leonoudakis et al., 2008; Yudowski et al., 2007). Newly inserted SEP-AMPA were readily distinguished as pronounced “spikes” of increased fluorescence intensity in videos of 1 minute duration. Blinded quantitative analysis of these videos was performed using MetaMorph image analysis software (see supplementary materials).

2.4. Excitotoxicity assay

Propidium iodide (PI) is a cell-impermeant fluorescent biomarker that stains DNA. Healthy viable cells exclude PI from their cytoplasm, whereas dead and dying cells take it up (Aras et al., 2008). Lactose dehydrogenase (LDH) is a stable cytosolic enzyme released during cell lysis from dead or dying cells and was used as a quantitative measure of neuron toxicity and death (Glass et al., 2004; Patel et al., 1993). TNF α (6 nM, R&D Systems) was added 15 min before kainate (KA, 20 μ M, Sigma), WIN (0.5 μ M) was added 30 min before TNF α and incubated for 24 hours. Nuclei of dead cells and total cells were stained with (PI) (5 μ g/ml, Sigma) and Hoechst (1 μ g/ml, Molecular Probes). Cultures were then washed 3 times by PBS and fluorescence images were collected. Images were thresholded to only show the nucleus by Metamorph software. Nuclei were counted automatically by the software and puncta ≥ 3 pixels were excluded. Percentage of cell death was calculated as (PI nuclei number)/(Hoechst nuclei number) $\times$ 100%. Immediately prior to staining, media in the cultures was collected for LDH quantification following the CytoTox 96 (Promega) protocol. All quantitative measures are expressed as means $\pm$ SEM. Statistical significance was determined by either one way ANOVA followed by Bonferroni's multiple comparison post test or unpaired t test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Expression of GluR1 and CB1 receptors on hippocampal neurons

In hippocampal neurons, GluR1-containing AMPARs co-localize with postsynaptic markers on somatic and dendritic plasma membranes (Beattie et al., 2002; Stellwagen et al., 2005) and CB1 has been detected in plasma membrane and cytosolic compartments of somato-dendritic regions as well as on axonal plasma membranes (Leterrier et al., 2006; McDonald et al., 2007). To confirm surface expression of CB1 in or near postsynaptic membrane in our hippocampal neuron cultures we have used immunofluorescence confocal microscopy methods which selectively detect surface receptors (see methods). We observed strong labeling of CB1 on axonal processes (Figure 1A and B, arrows) and colocalization of CB1 with PSD-95 staining on dendritic processes (Figure 1A, arrowheads). We also found CB1 is in close apposition to presynaptic synaptophysin staining (Figure 1B, arrowheads).

3.2. Cannabinoid receptor agonists inhibit surface AMPAR increase induced by TNF α

We next addressed the hypothesis that activation of CB1 reduces AMPAR surface expression. We first tested whether the CB1/CB2 agonist WIN55212-2 (WIN) had short term effects (minutes) on the constitutive level of AMPAR surface expression. WIN alone for 45 minutes did not significantly change the surface expression of GluR1 compared to control (Fig 2A and B) suggesting that short term receptor activation does not change constitutive AMPAR surface expression. In agreement with previous studies, 15 minutes of TNF α treatment alone increased steady state surface expression of GluR1-containing AMPARs compared to control on hippocampal neurons (Leonoudakis et al., 2008), but pretreatment with WIN for 30 minutes prior to 15 minutes of WIN + TNF α treatment blocked this increase in surface expression (Fig. 2. A, B). These results demonstrating a WIN-induced block of TNF α -induced AMPAR surface delivery led us to examine another exogenous cannabinoid receptor agonist, THC, and two endogenous cannabinoid agonists (endocannabinoids) (2AG) and anandamide (ANA). These agonists also blocked the TNF α -induced surface expression of GluR1-containing AMPARs [THC (106 $\pm$ 6.3%; * $p < 0.05$; n 80), 2AG (108 $\pm$ 3.5%; * $p < 0.05$; n 100) and anandamide (109 $\pm$ 3%; * $p < 0.05$; n 100)].

3.3. WIN inhibits the exocytosis increase of AMPAR induced by TNF α

The TNF α -induced increase in surface AMPARs is due to an increase in exocytosis of AMPARs from intracellular stores and is not the result of a decrease in surface receptor endocytosis (Stellwagen et al., 2005). Therefore, we hypothesized that WIN blocks the TNF α -induced increase of surface AMPARs by preventing an increase in the rate of AMPAR exocytosis in extrasynaptic plasma membrane. To test this hypothesis we used a quantitative video microscopy assay to measure the rate of AMPAR exocytosis events in real time (100 msec time resolution). Videos were taken after either 1) no treatment, 2) WIN treatment (between 15 and 45 minutes), or 3) TNF α treatment (between 15 and 45 minutes), or 4) WIN + TNF α treatment (between 15 and 45 minutes) (see supplementary materials). Figure 2C shows the graphic representation of the change in rate of exocytosis events (expressed as events/minute/ μm^2 of plasma membrane surface) between control vs. TNF α treatment and WIN vs. WIN + TNF α treatment. TNF α treatment in the absence of WIN doubles the rate of AMPAR exocytosis, while TNF α treatment does not increase the rate of exocytosis measured in WIN-pretreated neurons.

3.4. CB1, not CB2 inhibits the effects of TNF α on AMPAR expression

Since WIN and THC activate both CB1 and CB2, we pretreated neurons with specific CB1 or CB2 antagonists prior to CB1/CB2 agonist application to identify the CB receptor that might be responsible for preventing the TNF α -induced increase in AMPAR surface expression. As shown in Figure 3A, treatment with WIN blocked the TNF α -induced increase in cell surface AMPARs. This block was CB1-mediated, as it was reversed by the CB1-selective antagonist/inverse agonist SR141716A (SR1) (Hurst et al., 2006), but not the CB2-selective antagonist SR144528 (SR2) (Fig 3A). CB1 appeared to be constitutively active in this preparation, as treatment with SR1 enhanced the TNF α effect (Fig 3B). Therefore, we tested the effects of a neutral antagonist, VCHSR1 (VCH) (Hurst et al., 2006), and found that while VCH reversed the effects of WIN on AMPAR surface expression, it did not have intrinsic activity, as predicted for a neutral antagonist (Fig 3B).

3.5. CB1 activation mitigates the enhanced excitotoxic death caused by TNF α

Our results showing that CB1 activation prevents the rate of increase in TNF α -induced AMPAR exocytosis suggest that CB1 activation might protect neurons from TNF α -enhanced excitotoxic death. Thus, we challenged hippocampal neurons with TNF α (15 min pretreatment) and kainate [(KA): selective AMPA/kainate receptor agonist] and measured cell death 24 hrs later by propidium iodide (PI) uptake and Lactose dehydrogenase (LDH) release assays (Fig 4. A, B, and C). KA alone increased cell death compared to the control condition while TNF α alone did not show a change in cell death compared with control. Pretreatment of neurons with WIN prior to KA challenge did not protect neurons from cell death compared to the addition of KA alone as shown previously (Shen and Thayer, 1998). Pretreatment of the cultures with TNF α followed by the addition of KA significantly enhanced cell death compared with KA alone, suggesting that TNF α potentiates neuronal excitotoxicity. In contrast, preincubation with WIN prior to TNF α and KA addition significantly reduced the observed cell death compared to TNF α +KA co-application. Levels of cell death with KA alone or with WIN+TNF α +KA were not significantly different. Therefore, the potentiated KA excitotoxicity by TNF α is blocked by cannabinoid receptor activation.

4. Discussion

Our data is supportive of the hypothesis that activation of CB1 decreases TNF α -induced surface expression of AMPARs and protects against END in hippocampal cultures. CB1, AMPARs, and TNF α receptors are all expressed in dendrites in our cultured hippocampal

neurons where intracellular signaling between CB1 and TNF α receptors may compete to regulate AMPAR trafficking (Marsicano et al., 2003) as suggested in studies of cortical pyramidal neurons (Hill et al., 2007; Kim et al., 2006) and spinal cord neurons (Salio et al., 2002). Our data showing a correlation of CB1 signaling with a block of the TNF α -induced increase in AMPAR surface trafficking will guide future studies that will seek to identify intracellular signaling mechanisms driving this potential receptor cross-talk. Regardless of the precise mechanism, our results suggests a potential therapeutic benefit from CB1 activation in the vulnerable time-window immediately following CNS injury. Our studies show that pretreatment with CB1 agonists saves neurons in our excitotoxicity assay. How is this relevant to practical therapeutic intervention in neurotrauma patients since neurons lost in the “crush zone” of a spinal cord injury event, for example, would not likely be recovered by subsequent blocking of TNF α signaling? The answer comes from studies showing an extensive amount of slowly-spreading neuron death (spanning several days after the injury event) around the initial injury zone which presents a significant opportunity for therapeutic intervention targeting TNF α signaling (Ferguson et al., 2008; Gensel et al., 2006; Mihai et al., 2008). If, as we hypothesize, CB1 activation protects neurons through a mechanism that regulates AMPAR trafficking, how might this occur? The modulation of AMPARs by other GPCRs has been previously demonstrated. D1 dopamine receptor activation increases AMPAR surface expression (Gao et al., 2006; Sun et al., 2005), whereas activation of the metabotropic glutamate receptor (mGluR) decreases surface AMPARs (Davidkova and Carroll, 2007; Snyder et al., 2001; Xiao et al., 2001). PKA activation followed by phosphorylation of AMPARs is a gate to AMPAR surface delivery (Oh et al., 2006), and TNF α stimulation of neurons has been shown to increase PKA activity (Zhang et al., 2002a; Zhang et al., 2002b). It is possible that activation of CB1 may counter this TNF α -induced AMPAR surface delivery through an intracellular signaling mechanism that decreases PKA activation (Di Marzo et al., 2004; Kim et al., 2006; Wade et al., 2004).

CB1 appeared to be constitutively active in our hippocampal neuron preparation, as treatment with the CB1 antagonist/inverse agonist SR1 enhanced the TNF α effect. Therefore, we tested the effects of a neutral antagonist, VCHSR1 (Hurst et al., 2006), and found that while VCHSR1 reversed the effects of WIN on AMPAR surface expression, it did not have intrinsic activity, as would be predicted for a neutral antagonist. These data support a role for CB1 signaling in the modulation of TNF α -induced AMPAR surface-expression.

We hypothesized that the observed block of the TNF α -induced increase in surface AMPARs by cannabinoid receptor activation was mediated by a reduction in the rate of exocytosis. We previously reported that TNF α delivers AMPARs preferentially to extrasynaptic plasma membrane (Leonoudakis et al., 2008) and we have recently developed a technique that allows for the real-time observation AMPAR exocytotic events (Yudowski et al., 2007). Our current results using this new live imaging technique demonstrate that during CB1 activation TNF α treatment is unable to increase the rate of AMPAR exocytosis.

CB1 activation does not confer neuroprotection through non-AMPAR trafficking effects in our excitotoxicity assays. Our previous work showed that neurons pretreated with the AMPAR/KA receptor-specific antagonist CNQX before exposure to kainate were completely protected from excitotoxicity, indicating that the cell death produced by kainate requires AMPAR/KA receptor activity (Leonoudakis et al., 2008). These are in agreement with previous *in vitro* hippocampal studies showing the AMPAR dependence of TNF-potentiated neuron death (Bernardino et al., 2005). Our previous work as well as others' studies (Ogoshi et al., 2005; Stellwagen and Malenka, 2006) suggest that TNF α increases the surface delivery of Ca²⁺-permeable, GluR2-lacking AMPARs within 15 min of TNF α exposure. Ca²⁺-permeable AMPARs have been demonstrated to be major mediators of

excitotoxic cell death (Carriedo et al., 1996; Kwak and Weiss, 2006; Oguro et al., 1999). If these Ca^{2+} -permeable AMPARs are a significant component of the $\text{TNF}\alpha$ -induced surface AMPAR accumulation, then specifically blocking their activity, and thus Ca^{2+} influx through these channels, should reduce the extent of kainate-induced toxicity. Indeed, pretreatment of cultures with NASPM, a Ca^{2+} -permeable AMPAR antagonist, followed by KA plus $\text{TNF}\alpha$ produced no additional potentiation than NASPM+ KA (Leonoudakis et al., 2008). Our experiments show that WIN blocks this $\text{TNF}\alpha$ -induced increase in GluR1-containing AMPARs. We also show that KA alone, WIN+KA, and WIN+KA+ $\text{TNF}\alpha$ all induce a similar amount of cell death, whereas KA+ $\text{TNF}\alpha$ results in significantly more cell death. This suggests that WIN selectively mitigates excitotoxicity caused by a $\text{TNF}\alpha$ -induced increase in AMPAR surface expression, and not through other neuron death mechanisms (ie- programmed apoptotic pathways). The model supported by the data is one where WIN interferes with TNF -induced AMPAR exocytosis to reduce $\text{TNF}\alpha$ -potentiated excitotoxicity. We have previously shown this $\text{TNF}\alpha$ -potentiated excitotoxicity to be a significant proportion of the total measured *in vitro* excitotoxicity (Leonoudakis et al., 2008).

Normally, the surface expression of AMPARs and their delivery to synapses is tightly regulated. This precise regulation of surface AMPAR number and location as well as the control over glutamate availability prevent excitotoxicity (Malinow and Malenka, 2002). However, after injury, seizures, or during neurodegenerative disease there is an increase in the concentration of extracellular glutamate along with an increased potential for excessive AMPAR surface delivery and excitotoxicity (reviewed in (Leonoudakis et al., 2004; Pickering et al., 2005). Our previous biochemical and microscopic data examining total surface AMPAR levels over a 1 h time course of specific $\text{TNF}\alpha$ receptor activation is supportive of a maximum increase in total surface accumulation of calcium permeable AMPARs (CP-AMPA) at 15 min, which returns to baseline by 1 h (Leonoudakis et al., 2008). CP-AMPA are important functionally because they have stronger current passing capabilities compared with GluR2-containing AMPARs and are Ca^{2+} -permeable (Burnashev et al., 1992; Hollmann et al., 1991). When neurons are exposed to excess glutamate after neuronal damage, excessive surface expression of CP-AMPA induced by increased $\text{TNF}\alpha$ levels may lead to the abnormal influx of Ca^{2+} and overactivation of AMPARs resulting in potentiated excitotoxicity (Bernardino et al., 2005; Chao and Hu, 1994; Gelbard et al., 1993; Hermann et al., 2001). These data reveal a critical window of excitotoxic vulnerability caused by $\text{TNF}\alpha$. Inhibiting this activity through modulation of CP-AMPA trafficking (e.g., by CB1 activation) may reduce excitotoxicity after acute trauma or during neurological disorders.

Cannabinoid receptors protect neurons from excitotoxic stress and the mechanisms of this protection are diverse (Kim et al., 2006; Marsicano et al., 2003; Shen and Thayer, 1998, 1999). One of these mechanisms is believed to be mediated by the retrograde release of endogenous cannabinoids from the postsynaptic neuron to CB1 receptors located on presynaptic neurons [reviewed in (Chevalleyre et al., 2006)] but a role for post-synaptic CB1 signaling cannot be ruled out.

Our results provide the basis for further *in vitro* studies that will address the specific mechanisms underlying the CB1-mediated block of $\text{TNF}\alpha$ -induced increase in AMPAR surface expression as well as animal studies that will test the *in vivo* neuroprotective potential of CB1 activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

MEA is supported by grants from the NIH (DA09978, DA05274) and by The Forbes Norris ALS Center. EB is supported by grants from the NIH (MH067931 and NS038079) as well as by The Forbes Norris ALS Center, the California Pacific Medical Center Research Institute, and ALSA (starter grant 766). We thank Robert Kim and Sean Prasad for excellent technical assistance with the live imaging experiments. We thank Dr. Patricia Reggio (University of North Carolina, Greensboro) and Dr. Herb Seltzman (RTI) for providing VCHSR1 (DA039434).

Abbreviations

2AG	2-Arachidonoylglycerol
AMPArs	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ANA	anandamide
CB1	cannabinoid receptor 1
END	Excitotoxic Neuroinflammatory Death
GluR1	glutamate receptor 1
GPCR	G-protein-coupled receptor
KA	kainate
LDH	lactose dehydrogenase
SYN	synaptophysin
THC	Δ^9 -tetrahydrocannabinol
TNFα	Tumor necrosis factor α
WIN	WIN55212-2

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Figure 1. Surface CB1 is closely apposed to and colocalized with synaptic markers on dendrites of cultured hippocampal neurons

(A) The top three panels show confocal micrographs of the same field of view with either CB1, PSD-95, or CB1+PSD-95 (Merge) staining respectively. Co-staining of surface CB1 and internally localized PSD-95 demonstrates that cannabinoid receptors are expressed on dendrites of hippocampal neurons (arrow heads). Surface localization of CB1 on axonal processes which are devoid of PSD-95 is also shown (arrows) (B) The bottom three panels show another field of view with either CB1, the presynaptic marker synaptophysin (SYN), or CB1+SYN (Merge) staining respectively. Some CB1 receptors are colocalized with SYN (arrow heads), while others on the surface of axons are not overlapped with SYN (arrows). Scale bar, 5 μ m.

Figure 2. Cannabinoid receptor agonists block the steady state increase in AMPAR surface expression and the increased rate of AMPAR exocytosis induced by TNF α
 (A) Immunofluorescence microscopy demonstrating that TNF α treatment (15 min.) increases GluR1 surface staining on dendrites of hippocampal neurons compared to control, untreated neurons. Pretreatment of cultures with WIN blocked the GluR1 surface increase induced by TNF α . WIN alone (45 min.) did not change surface expression of GluR1. Scale bar, 10 μ m. (B) Immunofluorescent image quantification shows a significant increase in TNF α -induced surface GluR1 ($128 \pm 3.4\%$; *** $p < 0.001$) compared to control ($100 \pm 2.6\%$). Pretreatment of cells with WIN for 30 minutes blocked the TNF α -induced GluR1 surface increase ($100 \pm 3.2\%$; *** $p < 0.001$). WIN alone (45 min.) did not have a significant effect on GluR1 surface expression ($109 \pm 2.8\%$; $p > 0.05$). n = 100 neurons each condition from 5

independent experiments. (C) TNF α increases the *rate* of AMPAR exocytotic events on dendritic plasma membrane. Videos (1 minute duration and shown on-line in supplemental materials) of SEP-AMPA exocytosis from over 100 hippocampal neurons were collected after either, 1) no treatment, 2) TNF α treatment, 3) WIN treatment, or 4) WIN+TNF treatment. TNF α treatment significantly increases the rate of AMPAR exocytosis compared to control (TNF: $237.4 \pm 30.4\%$, $p < 0.01$; Control, $n=23$; TNF, $n=41$), while TNF α treatment in the presence of WIN did not change AMPAR exocytosis rates (WIN+TNF: $92.6 \pm 10.7\%$, $p > 0.05$; WIN, $n=16$; WIN+TNF, $n=40$) compared to WIN treatment alone.

Figure 3. The block of TNF α -induced increase of AMPAR surface expression requires CB1 but not CB2 activity

(A) CB1 antagonists SR1 and VCH antagonized the effect of WIN on TNF α -induced increases in surface GluR1 (TNF, $128 \pm 3.4\%$; TNF+WIN, $100 \pm 3.2\%$; SR1+WIN+TNF, $150 \pm 4.9\%$; VCH+WIN+TNF, $135 \pm 6.7\%$; compared to TNF+WIN *** $p < 0.001$). The CB2 antagonist SR2 did not (SR2+WIN+TNF, $109 \pm 4.5\%$). (B) Co-treatment of SR1 and TNF together induced a higher GluR1 increase than TNF alone (SR1+TNF, $164 \pm 5.4\%$; compared to TNF alone *** $p < 0.001$). SR2 and VCH showed no effect on TNF-induced surface GluR1 expression (SR1, $108 \pm 4.4\%$; SR2+TNF ($129 \pm 4.9\%$), VCH+TNF ($133 \pm 6.9\%$)). n 100 neurons each condition from 5 independent experiments.

Figure 4. Excitotoxicity is potentiated by TNF α and this potentiation blocked by cannabinoid receptor agonists

(A) Nuclear staining of dead cells (red) by PI and total cells (blue) by Hoechst demonstrates kainate (KA) induced excitotoxicity. TNF α potentiated the cell death caused by KA (TNF + KA). Pretreatment with WIN blocked the potentiated cell death induced by TNF α (WIN + TNF + KA). Arrowheads indicate dead cells. Scale bar, 200 μ m. (B) Quantification of image data in (A) demonstrate that KA induced more neuron death ($28.4 \pm 1.2\%$, * $p < 0.05$) than the control, untreated neurons ($16.0 \pm 1.1\%$). Adding TNF α before KA potentiated cell death ($43.6 \pm 4.2\%$, compared to KA ** $p < 0.01$). Pretreatment of cells with WIN significantly blocked cell death enhancement by TNF α ($31.2 \pm 2.0\%$, compared to TNF+KA ** $p < 0.01$). WIN alone ($17.5 \pm 2.6\%$) or TNF α alone ($14.9 \pm 2.5\%$) did not show a significant change in cell death compared to control. [n=25 images for each condition from 5 cultures].

(C) The amount of LDH released from dead or dying neurons was measured to confirm that WIN blocks TNF α -induced potentiation of excitotoxicity. TNF (85.1% $\pm$ 7.1); WIN (101.2% $\pm$ 1.6); KA (161.2% $\pm$ 12.3, compared to control * $p<0.05$); TNF+KA (230.7% $\pm$ 28.1, compared to KA ** $p<0.01$); WIN+KA (154.6% $\pm$ 12.9, compared to KA $p>0.05$) and WIN+TNF+KA (153.9% $\pm$ 8.3, compared to TNF+KA ** $p<0.01$). $n=21$ wells of cells for each condition from 7 cultures.