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Cannabinoids inhibit network-driven synapse loss between hippocampal neurons in culture

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Abstract

Dendritic pruning and loss of synaptic contacts are early events in many neurodegenerative diseases. These effects are dynamic and appear to differ mechanistically from the cell death process. Cannabinoids modulate synaptic activity and afford protection in some neurotoxicity models. We investigated the effects of cannabinoids on activity-induced changes in the number of synapses between rat hippocampal neurons in culture. Morphology and synapses were visualized by confocal imaging of neurons expressing DsRed2 and postsynaptic density protein 95 fused to enhanced green fluorescent protein (PSD95-GFP). Reducing the extracellular Mg^{2+} concentration to 0.1 mM for 4 hr induced intense synaptic activity that decreased the number of PSD95-GFP puncta by $45 \pm 13\%$. Synapse loss was an early event, required activation of NMDA receptors and was mediated by the ubiquitin-proteasome pathway. The cannabinoid receptor full agonist (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl] pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl) methanone monomethanesulfonate (WIN55,212-2; $EC_{50}=2.5 \pm 0.5$ nM) and the partial agonist Δ^9 -tetrahydrocannabinol (THC; $EC_{50}=9 \pm 3$ nM) inhibited PSD loss in a manner reversed by the CB1 receptor antagonist rimonabant. The protection was mimicked by inhibition of presynaptic Ca^{2+} channels and WIN55,212-2 did not prevent PSD loss elicited by direct application of glutamate, suggesting a presynaptic mechanism. Prolonged exposure to WIN55,212-2, but not THC, desensitized the protective effect. Treating cells that had undergone PSD loss with WIN55,212-2 reversed the loss and enabled recovery of a full complement of synapses. The modulation of synaptic number by acute and prolonged exposure to cannabinoids may account for some of the effects of these drugs on the plasticity, survival and function of neural networks.

Introduction

Δ^9 -tetrahydrocannabinol (THC), the principal psychoactive ingredient in marijuana, produces euphoria and relaxation and impairs motor coordination, time sense and short-term memory (Ameri, 1999). Clinically useful attributes of the cannabinoids (CBs) include their ability to produce analgesia, reduce chemotherapy-induced emesis, stimulate appetite, and attenuate seizures and spasticity (Croxford, 2003). The central actions of CBs are mediated by activation of CB1 receptors located primarily on presynaptic nerve terminals, where they interact with heterotrimeric G proteins (G_i/G_o) to inhibit neurotransmitter release via inhibition of voltage-gated Ca^{2+} channels and activation of K^+ channels (Howlett et al., 2004). Endocannabinoids (eCBs) are arachidonic acid derivatives produced in response to postsynaptic stimulation; they diffuse in a retrograde fashion across the synapse to act on presynaptic CB1 receptors to exert a similar inhibition of neurotransmitter release (Diana and Marty, 2004).

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Activation of CB1 receptors by either exogenous or endogenous ligands reduces the strength of the affected synapse. The eCB system mediates a long-term depression of projections from prefrontal cortex to nucleus accumbens neurons (Robbe et al., 2002) and long lasting autoinhibition of neocortical GABAergic interneurons (Bacci et al., 2004). In hippocampus, CBs inhibit GABA release from a subset of interneurons and inhibit glutamate release from principal neurons (Katona et al., 1999; Domenici et al., 2006). Thus, cannabinoids produce both rapid and long-term changes in synaptic transmission.

Cannabinoids protect hippocampal neurons from excitotoxicity (Shen and Thayer, 1998b) and from oxygen and glucose deprivation (Nagayama et al., 1999). Cannabinoids also provide protection in a variety of models of acute neurodegeneration in vivo (Nagayama et al., 1999; Panikashvili et al., 2001; van der Stelt et al., 2001). These effects have mostly been ascribed to inhibition of glutamatergic synaptic transmission (Shen et al., 1996; Gilbert et al., 2007) consistent with localization of CB1 receptors on glutamatergic nerve terminals (Domenici et al., 2006). Indeed, the eCB system appears to serve as an on-demand mechanism to reduce excitatory synaptic activity and thus protect from over-stimulation (Marsicano et al., 2003; Alger, 2004).

Changes in dendritic morphology accompany changes in synaptic strength (Harms and Dunaevsky, 2006). Indeed, stimuli that induce long term potentiation of synaptic transmission alter the shape of synaptic spines (Carlisle and Kennedy, 2005) and increase the number of synapses (Ma et al., 1999). Changes in dendritic morphology also appear in the early stages of neurodegenerative disorders (Sa et al., 2004). Dendritic spines undergo some of the earliest structural changes during excitotoxic injury to neurons (Olney et al., 1979) and changes in spine density are well documented in a variety of epilepsy models (Fiala et al., 2002). Synaptic loss is not necessarily a step on the path toward death, but instead may be a mechanism that enables the cell to adapt to excessive excitatory input (Finkbeiner et al., 2006; Waataja et al., 2008). If synaptic loss is a coping mechanism, then pharmacologic strategies to preserve network function may be important compliments to drugs that improve survival.

The postsynaptic density protein, PSD-95, is a scaffolding protein that anchors receptors and downstream signaling molecules to the postsynaptic density (Kim and Sheng, 2004) and is widely used as a marker for synaptic sites (Okabe et al., 1999). PSD-95 is removed from synapses in response to stimuli that induce long term depression of synaptic transmission (Colledge et al., 2003) and during epileptic activity (Zha et al., 2005).

Here, we studied changes in the number of synapses between hippocampal neurons identified by fluorescent puncta produced by expression of PSD-95 fused to enhanced green fluorescent protein (PSD-95-GFP). We show that intense excitatory synaptic activity reduced the number of synapses between hippocampal neurons in culture. We tested the hypothesis that cannabinoids prevent and reverse synaptic loss via presynaptic inhibition of glutamate release.

Methods

Materials

Materials were obtained from the following sources: the GFP-PSD-95 expression vector was kindly provided by Donald B. Arnold; expression vector for DsRed2 (pDsRed2-N1) from Clontech (Mountain View, CA); Rimonabant (SR141716) and THC from National Institute on Drug Abuse drug supply system (Bethesda, MD); fura-2 acetoxymethyl ester (AM), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and horse serum from Invitrogen (Carlsbad, CA); (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone monomethanesulfonate (WIN55,212-2), L-glutamate, (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]

cyclohepten-5,10-imine hydrogen maleate (MK801), Z-Leu-Leu-Leu-al (MG132) and all other reagents from Sigma (St. Louis, MO).

Cell culture

Rat hippocampal neurons were grown in primary culture as described previously (Shen and Thayer, 1998a) with minor modifications. Fetuses were removed on embryonic day 17 from maternal rats, anesthetized with CO₂, and sacrificed by decapitation. Hippocampi were dissected and placed in Ca²⁺ and Mg²⁺-free HEPES-buffered Hanks salt solution (HHSS), pH 7.45. HHSS was composed of the following (in mM): HEPES 20, NaCl 137, CaCl₂ 1.3, MgSO₄ 0.4, MgCl₂ 0.5, KCl 5.0, KH₂PO₄ 0.4, Na₂HPO₄ 0.6, NaHCO₃ 3.0, and glucose 5.6. Cells were dissociated by trituration through a 5 ml pipette and a flame-narrowed Pasteur pipette. Cells were pelleted and resuspended in DMEM without glutamine, supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively). Dissociated cells were then plated at a density of 15,000–20,000 cells/dish onto a 25-mm-round cover glass (#1) glued to cover a 19 mm diameter opening drilled through the bottom of a 35 mm Petri dish. The coverglass was coated with poly-D-lysine (0.1 mg/ml) and washed with H₂O. Neurons were grown in a humidified atmosphere of 10% CO₂ and 90% air (pH 7.4) at 37 °C, and fed at days 1 and 6 by exchange of 75% of the media with DMEM, supplemented with 10% horse serum and penicillin/streptomycin. Cells used in these experiments were cultured without mitotic inhibitors for a minimum of 12 days.

[Ca²⁺]_i imaging

Cells were loaded with 3 µM fura-2 AM at 37°C for 45 min in HHSS containing 0.5% BSA. Coverslips with cells were then transferred to a recording chamber, placed on the stage of an Olympus IX71 microscope (Melville, NY) and viewed through a 40 × objective. Excitation wavelength was selected with a galvanometer driven monochromator (8 nm slit width) coupled to a 75 W xenon arc lamp (Optoscan; Cairn Research Ltd, UK). [Ca²⁺]_i was monitored in a field of cells using sequential excitation of fura-2 at 340 and 380 nm to enable the calculation of ratio-based [Ca²⁺]_i every 1 s. Fluorescence images, 510/40 nm were projected onto a cooled CCD camera (Cascade 512B; Roper Scientific Inc.) controlled by MetaFluor software (Molecular Devices Corporation, Union City, CA, USA). Changes in fura-2 fluorescence were converted to [Ca²⁺]_i using the formula $[Ca^{2+}]_i = K_d \beta (R - R_{min}) / (R_{max} - R)$ where R is the 340/380 nm fluorescent intensity ratio. The dissociation constant (K_d) used for Fura-2 was 225 nM, and β was the ratio of emitted fluorescence at 380 nm in the absence and presence of calcium. R_{min}, R_{max}, and β were determined in ionomycin-permeabilized cells in calcium-free (1 mM EGTA) and 5 mM Ca²⁺ buffers. Values of R_{min}, R_{max}, and β were 0.443, 6.481 and 8.294, respectively.

Transfection

Rat Hippocampal neurons were transfected between 10 and 13 days in vitro using a modification of a protocol described previously (Waataja et al., 2008). Briefly, hippocampal cultures were incubated for at least 20 min in DMEM supplemented with 1 mM kynurenic acid, 10 mM MgCl₂, and 5 mM HEPES, to reduce neurotoxicity. A DNA/calcium phosphate precipitate containing 1 µg plasmid DNA per well was prepared, allowed to form for 30 min at room temperature and added to the culture. After a 90 min incubation, cells were washed once with DMEM supplemented with MgCl₂ and HEPES and then returned to conditioned media, saved at the beginning of the procedure. The transfection efficiency ranged from 10 to 12 %.

Confocal imaging

Forty-eight hrs after transfection neurons were transferred to the stage of a confocal microscope (Olympus Fluoview 300, Melville, NY) and viewed through a 60X oil-immersion objective (NA. 1.40). For experiments in which the same neurons were imaged before and after a 4 h interval, the locations of individual cells were recorded using micrometers attached to the stage of the microscope. Multiple optical sections spanning 8 μm in the z-dimension were collected (1 μm steps), and these optical sections were combined through the z-axis into a compressed z stack. GFP was excited at 488 nm with an argon ion laser and emission collected at 530 nm (10 nm band pass). The excitation and emission wavelengths for DsRed2 were 543 nm and >605 nm, respectively.

Image processing

To count and label PSD95-GFP puncta an automated algorithm was created using MetaMorph 6.2 image processing software described previously (Waataja et al., 2008). Briefly, maximum z-projection images were created from the DsRed2 and GFP image stacks. Next, a threshold set 1 s.d. above the image mean was applied to the DsRed2 image. This created a 1-bit image that was used as a mask via a logical AND function with the GFP maximum z-projection. A top-hat filter (80 pixels) was applied to the masked PSD95-GFP image. A threshold set 1.5 s.d. above the mean intensity inside the mask was then applied to the contrast enhanced image. Structures between 8 and 80 pixels (approximately 0.37 to 3.12 μm in diameter) were counted as PSDs. The structures were then dilated and superimposed on the DsRed2 maximum z-projection for visualization. PSD counts were presented as mean $\pm$ s.e.m. where n is the number of cells, each from separate cover glass over multiple cultures. We used Student's two-tailed *t*-test for single or ANOVA with Bonferoni's post test for multiple statistical comparisons.

Results

Visualizing changes in synaptic sites

We developed an imaging-based method to quantify the number of synaptic sites on hippocampal neurons in culture based on the detection of clusters of the scaffolding protein PSD95 fused to enhanced green fluorescent protein (PSD95-GFP) (Waataja et al., 2008). In Fig. 1 we show hippocampal neurons in culture that were transfected with expression plasmids for PSD95-GFP and DsRed2. Confocal imaging of transfected neurons was used to identify postsynaptic sites, as indicated by PSD95-GFP puncta (Fig. 1A) and to determine cell morphology, as revealed by red fluorescence (Fig. 1B). We quantified postsynaptic sites by using an image processing algorithm that applied the DsRed2 image as a binary mask to the PSD95-GFP image then identified puncta by locating intensity peaks of the appropriate size (approximately 0.37 to 3.12 μm in diameter). Fig. 1 C shows a processed image with counted puncta enlarged and superimposed on the DsRed2 image. PSD95-GFP puncta were localized on dendritic shafts and spines. Analysis of DsRed images of cells with spines, which were identified by an observer using morphological criteria (mushroom shape with length between 1 and 2 μm , base width between 0.4 and 0.7 μm and body width between 0.8 and 1.6 μm) prior to running the image processing algorithm to identify puncta, revealed that 87 ± 5 % of spines (n=8 cells) also contained PSD95-GFP puncta. We have shown previously that fluorescent puncta represent functional synapses as indicated by localized increases in $[\text{Ca}^{2+}]_i$ induced by NMDA, their close apposition to presynaptic sites of vesicular release labeled with FM4-64 and co-localization with NR2A and 2B immunoreactivity (Waataja et al., 2008). These results are consistent with previous studies showing that a PSD95-GFP fusion protein expressed in hippocampal pyramidal neurons was clustered at postsynaptic sites (Okabe et al., 1999).

Aberrant excitatory synaptic activity induces the loss of synaptic sites

Reducing the extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) in the medium bathing hippocampal cultures elicits an intense pattern of excitatory electrical activity that produces repetitive increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ spikes) (McLeod et al., 1998). When sustained, this aberrant pattern of neurotransmission results in synaptically mediated neuronal death. We imaged changes in $[Ca^{2+}]_i$ with fura-2 to verify that exposure to 0.1 mM $[Mg^{2+}]_o$ induced repetitive $[Ca^{2+}]_i$ spiking in the hippocampal cultures studied here (Fig. 1D–F). $[Ca^{2+}]_i$ spiking was driven by glutamatergic synaptic transmission as it was significantly reduced ($68 \pm 4\%$ inhibition, $n=41$) by treatment with the NMDA receptor antagonist MK801 (10 μ M) (Fig. 1D). Exposure to 0.1 mM $[Mg^{2+}]_o$ for 4 h decreased the number of PSD95-GFP puncta by $45 \pm 13\%$ ($n=8$) (Fig. 1G–H). Analysis of puncta localized to spines revealed that PSD loss accompanied spine loss. Following application of 0.1 mM $[Mg^{2+}]_o$ there was a $78 \pm 5\%$ decrease in PSDs on spines and an $80 \pm 4\%$ loss of spines ($n = 8$ cells). Pretreatment with MK801 (10 μ M) for 30 min prevented the 0.1 mM $[Mg^{2+}]_o$ -induced loss of PSDs (Fig. 1I), indicating that the 0.1 mM $[Mg^{2+}]_o$ -induced loss of PSD95-GFP is dependent on the NMDA receptor. NMDA receptor activation can induce PSD95 degradation via the ubiquitin-proteasome pathway. We found that intense excitatory synaptic activity also targets PSD95-GFP to the proteasome as indicated by protection from 0.1 mM $[Mg^{2+}]_o$ -induced synapse loss by the proteasome inhibitor MG132 (50 μ M) (Fig. 1I). These results suggest that intense excitatory synaptic activity causes the loss of synaptic connections.

We next determined the time course for changes in the number of PSD95-GFP puncta and cell survival. Survival was defined as the retention of cytoplasmic DsRed2 protein. As shown in Fig. 2A, cell survival was stable for 24 hrs under control conditions and the number of PSD95-GFP puncta increased initially. The precise mechanism for this increase is not known, although the data in Figure 2C indicated that it develops in a graded fashion. It was triggered by the media exchange as experiments in which cells remain in growth media showed a significantly smaller increase ($6 \pm 5\%$ vs. $49 \pm 10\%$). New synapse formation required protein synthesis because it was inhibited by cycloheximide (10 μ M) ($-2 \pm 13\%$) but did not require synaptic activity as indicated by a lack of effect of tetrodotoxin (TTX) ($40 \pm 18\%$). Exposure to 0.1 mM $[Mg^{2+}]_o$ caused a significant loss of PSD95-GFP puncta by 1 hr ($-30 \pm 4\%$, $p<0.05$, $n=8$) and the magnitude of the loss increased to $-39 \pm 15\%$ by 8 hrs. In contrast, no cells died after 1 hr exposure to 0.1 mM $[Mg^{2+}]_o$ and the number of dead cells slowly increased to $29 \pm 18\%$ after 8 hrs in 0.1 mM $[Mg^{2+}]_o$ and increased further to $71 \pm 18\%$ death by 24 hrs ($n = 7$). Thus, 0.1 mM $[Mg^{2+}]_o$ -induced synaptic activity produces a time dependent loss of PSDs that precedes overt cell death.

Cannabinoid receptor agonists prevent 0.1 mM $[Mg^{2+}]_o$ induced PSD loss

We have previously shown that cannabinoid receptor agonists inhibit both the frequency of $[Ca^{2+}]_i$ spiking induced by reducing $[Mg^{2+}]_o$ and glutamatergic EPSCs elicited by stimulation of the presynaptic neuron (Shen et al., 1996). We tested the effect of cannabinoid agonists on 0.1 mM $[Mg^{2+}]_o$ -induced PSD loss. As shown in Fig. 3, the cannabinoid receptor full agonist WIN55,212-2 (100 nM) reduced PSD loss by $81 \pm 25\%$ ($n=7$) (Fig. 3A, B, E). The cannabinoid receptor partial agonist THC, at the same concentration, reduced PSD loss by $66 \pm 11\%$ (Fig. 3C, D, E). The selective CB1 receptor antagonist rimonabant completely blocked the actions of both drugs (Fig. 3E). Rimonabant alone did not affect 0.1 mM $[Mg^{2+}]_o$ -induced PSD loss ($-46 \pm 16\%$, $p<0.01$, $n=4$). The cannabimimetic drugs produced a concentration dependent protection from the SD loss induced by 0.1 mM $[Mg^{2+}]_o$ (Fig. 4). THC reduced the loss of PSD95-GFP puncta with an EC_{50} of 9 ± 3 nM. WIN55,212-2 was more potent than THC with an EC_{50} of 2.5 ± 0.5 nM.

WIN55,212-2 acts presynaptically

Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission by acting presynaptically to inhibit the release of glutamate (Shen et al., 1996). To test whether the effects of cannabimimetic drugs on synaptic sites were mediated via actions at pre- or postsynaptic targets we examined the effects of WIN55,212-2 on changes in PSDs evoked by treatment with glutamate. Application of glutamate (4 h, 100 μ M) to cultured hippocampal neurons reduced the number of PSD95-GFP puncta by 63 ± 14 % (Fig. 5A). In contrast to the effects of cannabimimetics on the synaptically-mediated PSD loss induced by 0.1 mM $[Mg^{2+}]_o$, WIN55,212-2 did not prevent the PSD loss elicited by the direct application of glutamate (Fig. 5A). Thus, cannabinoid receptor agonists act presynaptically to inhibit 0.1 mM $[Mg^{2+}]_o$ -induced PSD loss.

A presynaptic site of action for the CB1 agonists would be consistent with an inhibition of N- and P/Q- type Ca^{2+} channels (Shen and Thayer, 1998a). We tested this hypothesis by treating hippocampal cultures for 5 min with 1 μ M ω -conotoxin GVIAz, a selective blocker of N-type Ca^{2+} channels, prior to application of 0.1 mM $[Mg^{2+}]_o$. ω -conotoxin GVIA completely protected hippocampal neurons from 0.1 mM $[Mg^{2+}]_o$ induced PSD loss ($n = 10$) (Fig. 5B). Pretreatment with 1 μ M ω -agatoxin IVAz, a selective blocker of P/Q-type Ca^{2+} channels, for 20 min also significantly reduced the loss of PSDs. Neither channel blocker significantly affected the number of PSDs under control conditions (Fig. 5B). Thus, blocking presynaptic Ca^{2+} channels required for glutamate release prevents the loss of postsynaptic sites induced by intense excitatory synaptic activity.

Prolonged exposure to WIN55,212-2 diminishes the protection of PSDs by cannabinoids

Prolonged exposure to WIN55,212-2 or THC desensitized CB1 receptor-mediated inhibition of synaptic transmission (Kouznetsova et al., 2002; Lundberg et al., 2005). Here, we investigated whether prolonged exposure to cannabinoid receptor agonists diminished the protective effect of cannabimimetic drugs on 0.1 mM $[Mg^{2+}]_o$ -induced loss of PSDs. When cultures were pretreated with 1 μ M WIN55,212-2 for 24 hr, the protection afforded by cannabimimetic drugs was reduced. In cultures pretreated with WIN55,212-2, subsequent treatment with 0.1 mM $[Mg^{2+}]_o$ in the continued presence of 100 nM WIN55,212-2 resulted in a 49 ± 14 % decrease in PSDs. Similarly, in cells pretreated with WIN55,212-2 (1 μ M) for 24 h subsequent application of 0.1 mM $[Mg^{2+}]_o$ and 100 nM THC resulted in a 18 ± 8 % loss of PSDs (Fig. 6), which was not significantly different from the loss induced by 0.1 mM $[Mg^{2+}]_o$ in the absence of drug. Prolonged exposure to a maximally effective concentration of THC produces less functional desensitization of CB1-mediated inhibition of excitatory synaptic transmission than that produced by a maximally effective concentration of WIN55,212-2 (Lundberg et al., 2005). Thus, we examined the possibility that the reduced 0.1 mM $[Mg^{2+}]_o$ -induced PSD loss produced by THC might be more sustained than that of WIN55,212-2. In cultures pretreated with 1 μ M THC for 24 h, subsequent treatment with 0.1 mM $[Mg^{2+}]_o$ in the continued presence of 100 nM THC resulted in a 58 ± 29 % increase in PSDs, significantly different from the loss seen in the absence of drug (Fig. 6). Thus, even after 24 h pretreatment THC significantly attenuated 0.1 mM $[Mg^{2+}]_o$ -induced PSD loss.

Cannabinoids reverse synaptically driven synapse loss

If PSD loss proceeds via a path that is independent from that leading to cell death, then synapse loss might be reversible. We next examined whether WIN55,212-2 could reverse synaptically driven PSD loss following exposure to 0.1 mM $[Mg^{2+}]_o$. Hippocampal neurons expressing PSD95-GFP and DsRed2 were exposed to 0.1 mM $[Mg^{2+}]_o$ for 1 h which produced a 39 ± 6 % loss of PSDs (Fig. 7A, B). WIN55,212-2 (100 nM) was added at this time and 3 and 23 h following application of the drug in the continued presence of 0.1 mM $[Mg^{2+}]_o$ the cells were imaged again (4 and 24 hrs elapsed time). After 3 h in the presence of WIN55,212-2, neurons

recovered their PSDs ($10 \pm 10\%$ increase relative to before $0.1\text{ mM } [\text{Mg}^{2+}]_o$, $p < 0.001$, $n = 8$) (Fig. 7B). This recovery persisted for 23 h following application of WIN55,212-2. Synapse loss induced by 1 hr treatment with $0.1\text{ mM } [\text{Mg}^{2+}]_o$ was also reversed by returning $[\text{Mg}^{2+}]_o$ to 0.9 mM (data not shown). Interestingly, hippocampal neurons that were exposed to $0.1\text{ mM } [\text{Mg}^{2+}]_o$ for 4 h before addition of WIN55,212-2 did not recover their PSDs ($35 \pm 11\%$ decrease relative to before $0.1\text{ mM } [\text{Mg}^{2+}]_o$, $n = 4$) (Fig. 7C). Therefore, there is a window of at least 1 hr but less than 4 hr during which activation of CB1R can reverse synaptic loss resulting from epileptic patterns of synaptic activity.

Discussion

We used an in vitro model to quantify changes in the number of individual synapses in a synaptic network over time. The intense synaptic activity evoked by reducing $[\text{Mg}^{2+}]_o$ to 0.1 mM decreased the number of PSD95-GFP puncta by $45 \pm 13\%$. This observation is consistent with previous reports showing a decrease in spine density in hippocampal neurons exposed to glutamatergic agonists or epileptiform activity (Muller et al., 1993; Colledge et al., 2003; Zha et al., 2005). Cannabinoids acted potently on presynaptic CB1 receptors to prevent or even reverse PSD loss that resulted from intense synaptic activation of NMDA receptors. Thus, presynaptic inhibition attenuated synaptic transmission sufficient to prevent long term changes in the number of synapses. Cannabinoid modulation of synapse loss desensitized, raising interesting questions about the changing role of the endocannabinoid system in neuroprotection following chronic exposure to drugs. PSD loss is an early event in neurodegenerative disease; its reversal by cannabinoids has implications for protection of network function, modulation of synaptic plasticity and regulation of neuronal survival.

We used PSD-95, a protein that serves as a scaffold for assembling signaling molecules at excitatory synapses, as a marker for synapses between hippocampal neurons in culture. Glutamate produces a local $[\text{Ca}^{2+}]_i$ increase at PSD95-GFP puncta, these puncta are in close apposition to presynaptic sites of vesicular release and they co-localize with NR2A and 2B immunoreactivity (Waataja et al., 2008). Thus, PSD95-GFP puncta represented functional postsynaptic sites. The formation and loss of synaptic puncta were highly dynamic, consistent with previously reported high turnover rates for synapses in culture (Okabe et al., 1999). The increase in synaptic number induced by media exchange required protein synthesis. Thus, the increase was not an imaging artifact, but instead was a physiological response to the experimental protocol. The increase did not impede quantitative analysis and statistical testing of hypotheses because it was reproducible and in the opposite direction from activity-induced changes. Epileptic activity induced a robust loss of puncta that was highly reproducible and statistically different from the control conditions. This loss was seen in PSDs located on dendritic shafts and spines. Zha et al (2005) have described a similar activity-induced loss of PSDs on spines but in their study TTX induced a loss of PSDs on dendritic shafts. The dissociated cultures used in this study were derived from fetal animals whereas Zha et al (2005) used postnatal slice cultures. Overall, our data are in good agreement with previous studies that found a loss of excitatory synapses associated with epileptic activity (Muller et al., 1993; Colledge et al., 2003; Zha et al., 2005); we extended these previous studies to show pharmacological modulation of the process by cannabinoids. This assay was particularly well-suited to detect the recovery of synapses following loss because quantitative data could be obtained from the same cell over time. Loss of synaptic puncta was dependent on activation of NMDA receptors and mediated by the ubiquitin-proteasome pathway, in good agreement with a study showing that PSD-95 is ubiquitinated and rapidly removed from synaptic sites in response to NMDA receptor activation (Colledge et al., 2003). Thus, intense synaptic activity leads to proteasome-mediated loss of PSD95-GFP from synaptic sites.

Loss of postsynaptic proteins and dendritic spines are early events in many neurodegenerative disorders (Gyllys et al., 2004). Loss of synaptic terminals in the brains of Alzheimer's patients more closely correlates with decreased cognitive function than cell death, supporting the hypothesis that disappearance of synapses is a key event in early cognitive decline (Terry et al., 1991). Here we showed that intense glutamatergic synaptic activity decreased the number of synapses long before cells succumbed to excitotoxic death. This loss was reversible and mediated by the ubiquitin-proteasome pathway, attributes not generally associated with cell death pathways. Synapse loss may be a coping mechanism that is distinct from cell death processes because loss of PSDs appears to protect neurons from over-stimulation and subsequent toxicity (Waataja et al., 2008). Loss of synaptic function as a result of degenerative disease, pharmacologic block of NMDA receptors or cannabinoid inhibition of glutamate release, all produce cognitive impairment and all appear to improve neuronal survival during excitotoxic insult. Thus, neuroprotective strategies need to balance protecting PSDs, which can exacerbate over-stimulation of the postsynaptic cell, with allowing synapse down regulation, which may improve survival at the cost of reduced participation in the synaptic network.

Here we showed for the first time that inhibiting presynaptic Ca^{2+} channels either directly or by activation CB1 receptors prevented synapse loss resulting from epileptic activity. Ca^{2+} channel blockers are neuroprotective in some models of neurodegeneration (Valentino et al., 1993) and cannabinoids have antiepileptic properties (Croxford, 2003; Marsicano et al., 2003). However, this is the first report to show that either class of compounds protects network integrity from an excitotoxic insult. As a neuroprotective strategy drugs that inhibit glutamate release have the advantage of both preventing synapse loss and improving survival. A disadvantage of this approach is the potential for psychoactive side effects.

Cannabinoids acted potently on presynaptic CB1 receptors to prevent excitotoxic synapse loss. These actions were in good agreement with previous reports from our laboratory (Shen et al., 1996; Shen and Thayer, 1999) and others (Hajos et al., 2001) showing cannabinoid inhibition of glutamatergic synaptic transmission. The reversal of the protective actions of the cannabinoids by rimonabant suggests that this drug might modify seizure threshold and network function in circuits with strong eCB tone (Marsicano et al., 2003). Interestingly, THC completely blocked PSD loss even though it reduces synaptic activity by less than 50 % (Shen and Thayer, 1999), consistent with the idea that attenuation without complete block is sufficient to protect synaptic sites. This finding and the observation that the network displays a modest level of synaptic activity under control conditions suggest that the loss of PSDs requires sustained intense synaptic activity. Reducing $[\text{Mg}^{2+}]_o$ to 0.1 mM evoked an aberrant pattern of synaptic activity that approximates epileptic discharges (McLeod et al., 1998). Down regulation of synapses may underlie long term depression of synaptic transmission (Colledge et al., 2003) in which case the threshold level of activity to induce PSD loss would presumably not be toxic. Perhaps the dramatic synapse loss described here in response to intense stimulation and the sustained moderate activity required to induce LTD lie on a continuum of activity that produces corresponding changes in synaptic input to stabilize neuronal excitability (Desai, 2003).

The cannabinoid receptor agonist WIN55,212-2 reversed the synaptic loss induced by 0.1 mM $[\text{Mg}^{2+}]_o$ in the continued presence of the excitatory stimulus. This observation suggests that pharmacologic treatments might be used to improve or protect network function independent of treating the underlying cause of the synaptic loss. We speculate that protecting synapses might be important to pursue in parallel to strategies aimed at improving survival. Thus, the effective protection of synapses by cannabinoid agonists and calcium channel blockers suggests that network protection is in principle a reasonable approach. It will be interesting to evaluate other targets for synapse protection such as inhibition of postsynaptic proteolysis. If

synapse loss is indeed a coping strategy then synapse protection may increase the risk of excitotoxicity and thus, will need to be part of an approach that also improves survival. Our results also indicate that there is a window during which synapse loss can be reversed. The cell culture model employed for these studies is highly dynamic and would appear to undergo changes more rapidly than synapses studied in vivo (Grutzendler et al., 2002). Thus, the opportunity to protect network function may be longer in vivo where both loss and recovery may proceed on a slower time scale. Clearly more in vivo studies of synaptic loss and recovery are warranted.

Cannabinoid-induced presynaptic inhibition of glutamatergic neurotransmission desensitizes after prolonged exposure to CB1 receptor agonists (Lundberg et al., 2005). Prolonged exposure to WIN55,212-2 diminished the protective effect of cannabimimetic drugs. However, even when tested at a maximally effective concentration, the desensitization produced by THC was less pronounced than that produced by WIN55,212-2. Thus, THC exerted a longer lasting modulation of synaptic function than the more potent and efficacious agonist. Interestingly, chronic WIN55,212-2 treatment did not itself affect the number of synapses (Fig. 6), but instead prevented subsequent protection afforded by CB1 receptor agonists (Fig. 6). Thus, prolonged exposure to cannabinoid agonists might diminish on-demand protection of synapses mediated by eCBs. These observations suggest that therapeutic strategies that enhance eCB function without chronic CB1 receptor activation, for example by inhibiting metabolism, might prove most effective for long-term protection of synaptic function. This approach would also avoid the desensitization that accompanies prolonged exposure to agonists.

In conclusion, we showed that intense excitatory synaptic activity decreases synapses between hippocampal neurons and that attenuation of this activity by cannabinoids prevented synapse loss. Excitatory neurons maintain a precarious balance between full integration into excitatory networks, with the associated risk of over-stimulation, and withdrawal from the network to increase survival, with the associated loss of network function. Modulation of presynaptic CB1 receptors either directly or through modulation of the eCB system may prove to be a useful pharmacologic approach for maintaining this balance during neurodegenerative disease.

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Abbreviations

PSD95-GFP

postsynaptic density protein 95 fused to enhanced green fluorescent protein

[Mg²⁺]_o

extracellular Mg²⁺ concentration

WIN55

212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl] pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone monomethanesulfonate

THC

Δ⁹-tetrahydrocannabinol

rimonabant

	N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide
NMDA	N-methyl-D-aspartate
CB	cannabinoid
eCB	endocannabinoid
MK801	(5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate
MG132	Z-Leu-Leu-Leu-al
DMEM	Dulbecco's modified Eagle's Medium
HHSS	HEPES buffered Hank's salt solution
BSA	bovine serum albumin
TTX	tetrodotoxin

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Fig. 1.

Aberrant excitatory synaptic activity induces NMDA receptor-dependent loss of PSD95-GFP puncta. Confocal laser scanning microscopy was used to image a cultured hippocampal neuron expressing PSD95-GFP (A) and DsRed2 (B). Maximum z-projections (8 steps, 1 μ m/step) were created from the PSD95-GFP z-series (A) and the DsRed2 z-series (B). C, After enhancing contrast, those puncta reaching an intensity threshold and meeting size criteria were enlarged to improve visualization and superimposed on the DsRed2-defined mask (red). The insets are enlarged images of the boxed region in (A). Image processing is described in Methods. Images of PSD95-GFP expressing cells in Figs 1G-H, 2, 3 and 7 are presented in this processed format. Scale bars, 10 μ m. D, $[Ca^{2+}]_i$ spiking was elicited by superfusion with 0.1 mM $[Mg^{2+}]_o$ and measured by fura-2-based digital imaging. Traces from the 4 cells shown in E-F displayed synchronized $[Ca^{2+}]_i$ spiking. Treatment with MK801 (10 μ M) for 30 min inhibited $[Ca^{2+}]_i$ spiking. $[Mg^{2+}]_o$ was reduced to 0.1 mM for the entire recording and drug was added by superfusion at the time indicated by the horizontal bar. E-F, Pseudocolor representations of $[Ca^{2+}]_i$ were derived from fura-2-based digital images. Image pairs were collected at the times indicated in D and scaled as shown. G-H, Confocal images were collected from hippocampal cultures 48 h after transfection with expression vectors for PSD95-GFP and DsRed2. Representative set of images show the same cell before (G) and after 4 h treatment with 0.1 mM $[Mg^{2+}]_o$ (H). Scale bar, 10 μ m. I, Bar graph shows that 4 h treatment with 0.1 mM $[Mg^{2+}]_o$ significantly reduced the number of synaptic sites. Pretreatment with 10 μ M MK801 for 30 min or 50 μ M MG132 for 1 hr prior to and during exposure to 0.1 mM $[Mg^{2+}]_o$ attenuated

the loss of PSDs. Data are expressed as mean $\pm$ SEM. *, $p < 0.01$ relative to 0.9 mM $[\text{Mg}^{2+}]_o$, #, $p < 0.05$ relative to untreated 0.1 mM $[\text{Mg}^{2+}]_o$ (ANOVA with Bonferroni post test).

Fig. 2.

Time and activity dependent changes in synapses between hippocampal neurons. Confocal images were collected from hippocampal cultures 48 h after transfection with expression vectors for PSD95-GFP and DsRed2. Representative images were collected at the times indicated from a cell under control conditions (A, 0.9 mM $[Mg^{2+}]_o$) and during intense excitatory synaptic activity (B, 0.1 mM $[Mg^{2+}]_o$). Scale bars, 10 μ M. C, Graph shows time-dependent changes in the number of PSD95-GFP puncta (PSDs, filled symbols) and cell viability (Cell Survival, open symbols) for cells bathed in 0.9 mM $[Mg^{2+}]_o$ (squares, solid lines) and 0.1 mM $[Mg^{2+}]_o$ (circles, dashed lines). Data are expressed as mean $\pm$ SEM. #, $p < 0.01$ relative to cells counted prior to the addition of 0.1 mM $[Mg^{2+}]_o$ (0 hr); *, $p < 0.01$ relative to PSDs counted prior to the addition of 0.1 mM $[Mg^{2+}]_o$ (0 hr). ANOVA with Bonferroni post test.

Fig. 3. Cannabinoid receptor agonists prevent PSD loss. Confocal images were collected from hippocampal cultures 48 h after transfection with expression vectors for PSD95-GFP and DsRed2. A–D, Representative images show PSD95-GFP puncta before (0 h; A, C) and after 4 h (B, D) in 0.1 mM $[Mg^{2+}]_o$. Cultures were treated with 100 nM WIN55,212-2 (Win, A–B) or 100 nM THC (C–D) 5 min prior to and during exposure to 0.1 mM $[Mg^{2+}]_o$. Scale bars, 10 μm. E, Bar graph summarizes the effects of cannabinoid agonists on changes in PSD95-GFP puncta (PSDs) after 4 h treatment under control (0.9 mM $[Mg^{2+}]_o$, open bars) or stimulated (0.1 mM $[Mg^{2+}]_o$, solid bars) conditions. Treatment with 100 nM WIN55,212-2 (Win) or 100 nM THC prevented the loss of PSDs. The protection afforded by both agonists was blocked

in the presence of CB1 receptor antagonist rimonabant (rim, 100 nM). Data are mean ! SEM. * $p < 0.01$ relative to 0.9 mM $[Mg^{2+}]_o$; **, $p < 0.01$ relative to untreated 0.1 mM $[Mg^{2+}]_o$; #, $p < 0.01$ relative to WIN55,212-2 or THC plus 0.1 mM $[Mg^{2+}]_o$ (ANOVA with Bonferroni post-test).

Fig. 4.

Concentration-dependent protection of PSDs by cannabinoids. 48 h after transfection with expression vectors for PSD95-GFP and DsRed2 hippocampal cultures were treated with the indicated concentration of WIN55,212-2 (solid squares) or THC (solid circles) 5 min prior to and during exposure to 0.1 mM $[Mg^{2+}]_o$. The number of PSD95-GFP puncta were counted before and 4 h after treatment with 0.1 mM $[Mg^{2+}]_o$. The mean $\pm$ SEM of the net change in PSD95-GFP puncta 4 hrs after treatment with 0.1 mM $[Mg^{2+}]_o$ are plotted for the drug concentrations indicated ($n \geq 3$ for each data point). Curves were fit by a logistic equation of the form % PSD change = $[(A1 - A2)/(1 + (X/EC_{50})^p)] + A2$ where X = drug concentration, $A1 = -45 \pm 13$ % PSD change in 0.1 mM $[Mg^{2+}]_o$ without drug, $A2$ = % PSD change in 0.1 mM $[Mg^{2+}]_o$ at a maximally effective drug concentration and p = slope factor. EC_{50} values were calculated using a nonlinear, least-squares curve fitting program (Origin 6.0, OriginLab Corp.) and are expressed as mean $\pm$ SEM. EC_{50} , $A2$ and p for THC were 9 ± 3 nM, 56 ± 4 % and 1.6 ± 1.1 , respectively. EC_{50} , $A2$, and p for Win55,212-2 were 2.5 ± 0.5 nM, 63 ± 13 % and 1.7 ± 0.7 , respectively.

Fig. 5.

WIN55212,2 acts presynaptically. WIN55,212-2 did not prevent the loss of PSDs induced by the direct application of glutamate (A). Confocal images were collected from hippocampal cultures 48 h after transfection with expression vectors for PSD95-GFP and DsRed2. Bar graph summarizes the effects of Win55,212-2 (Win) on changes in PSD95-GFP puncta (PSDs) evoked by 4 h treatment in the absence (Control) or presence of 100 μ M glutamate (Glu). WIN55,212-2 (100 nM) was applied 5 min prior to and during treatment with glutamate. Data are expressed as mean $\pm$ SEM. * p <0.01 relative to control. Inhibition of voltage-gated Ca^{2+} channels prevents 0.1 mM $[Mg^{2+}]_o$ -induced synapse loss (B). Bar graph summarizes the effects of Ca^{2+} channel toxins on changes in PSD95-GFP puncta (PSDs) after 4 h treatment under

control (0.9 mM $[Mg^{2+}]_o$, open bars) or stimulated (0.1 mM $[Mg^{2+}]_o$, solid bars) conditions. Cultures were treated with 1 μ M -conotoxin GVIA (ω -CgTx) for 5 min or 1 μ M -agatoxin IVA (ω -AgTx) for 20 min prior to reducing the $[Mg^{2+}]_o$. Data are expressed as mean $\pm$ SEM. *, $p < 0.01$ relative to 0.9 mM $[Mg^{2+}]_o$; #, $p < 0.01$ relative to untreated 0.1 mM $[Mg^{2+}]_o$ (ANOVA with Bonferroni post-test).

Fig. 6.

Prolonged exposure to WIN55,212-2 diminishes the protection of PSDs by cannabinoid agonists. Confocal images were collected from hippocampal cultures 48 h after transfection with expression vectors for PSD95-GFP and DsRed2. Bar graph summarizes the effects of cannabinoid agonists on changes in PSD95-GFP puncta (PSDs) after 4 h treatment under control (0.9 mM [Mg²⁺]_o, open bars) or stimulated (0.1 mM [Mg²⁺]_o, solid bars) conditions. Treatment with either 100 nM WIN55,212-2 (Win) or 100 nM THC 5 min prior to and during the experiment is indicated below each set of paired bars. Shading indicates experiments in which the cultures were pretreated with 1 μM WIN55,212-2 (Win) or 1 μM THC for the 24 hr preceding the initial PSD count. Data are expressed as mean ± SEM. *, $p < 0.01$ relative to 0.9 mM [Mg²⁺]_o; **, $p < 0.01$ relative to untreated 0.1 mM [Mg²⁺]_o; #, $p < 0.05$ relative to WIN55,212-2 plus 0.1 mM [Mg²⁺]_o; §, $p < 0.05$ relative to THC plus 0.1 mM [Mg²⁺]_o; †, $p < 0.05$ relative to 24 hr pretreatment with WIN55,212-2 followed by THC plus 0.1 mM [Mg²⁺]_o (ANOVA with Bonferroni post-test).

Fig. 7.

Cannabinoids reverse synaptically driven synapse loss. Representative images display labeled PSDs on a neuron expressing PSD95-GFP and DsRed2 before (0 h) and after 1, 4 and 24 hr treatment with 0.1 mM $[Mg^{2+}]_o$. WIN55,212-2 (100 nM) was added after 1 hr and additional images collected after 3 and 23 hrs in the presence of both WIN55,212-2 and 0.1 mM $[Mg^{2+}]_o$ (4 and 24 h elapsed time). Note that PSDs lost after 1 h exposure to 0.1 mM $[Mg^{2+}]_o$ recovered following addition of WIN55212-2 (arrow heads). Not all PSDs that appeared during recovery were found at sites labeled prior to 0.1 mM $[Mg^{2+}]_o$ treatment. Scale bars, 10 μ m. (B–C) Line graphs illustrate time-dependent changes in the number of PSDs under control conditions (solid squares) and in 0.1 mM $[Mg^{2+}]_o$ (solid circles). WIN55212,2 (dashed

line, open triangle) reversed synapse loss when added after 1 h (B) but failed to affect PSD number after a 4 h exposure to 0.1 mM $[\text{Mg}^{2+}]_o$ (C) (dashed line, open diamond). $n \geq 8$ for each data point. *, $p < 0.01$ relative to 0.1 mM $[\text{Mg}^{2+}]_o$ at the same time.