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Molecular Mechanisms of Cannabinoid Protection from Neuronal Excitotoxicity

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ABSTRACT

Cannabinoids protect neurons from excitotoxic injury. We investigated the mechanisms involved by studying *N*-methyl-D-aspartate (NMDA) toxicity in cultured murine cerebrocortical neurons in vitro and mouse cerebral cortex in vivo. The cannabinoid agonist *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl]-(1-naphthalenyl)-methanone mesylate [*R*(+)-Win 55212] reduced neuronal death in murine cortical cultures treated with 20 μM NMDA, and its protective effect was attenuated by the CB1 cannabinoid receptor (CB1R) antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-cichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A). Cultures from CB1R-knockout mice were more sensitive to NMDA toxicity than were cultures from wild-type mice. The in vitro protective effect of *R*(+)-Win 55212 was reduced by pertussis toxin, consistent with signaling through CB1R-coupled G-proteins. The nitric-oxide synthase (NOS) inhibitors 7-nitroindazole (7-NI) and *N*-ω-nitro-L-arginine methyl ester also reduced NMDA toxicity. In addition, CB1R and neuronal NOS were coexpressed in cultured cortical neurons, suggesting that cannabinoids might reduce NMDA toxicity by interfering with the generation of NO. NOS activity in cerebral cortex was higher in CB1R-knockouts than in wild-type mice, and 7-NI reduced NMDA lesion size. *R*(+)-Win 55212 inhibited NO production after NMDA treatment of wild-type cortical neuron cultures, measured with 4-amino-5-methylamino-2′,7′- difluorofluorescein diacetate, and this effect was reversed by SR141716A. In contrast, *R*(+)-Win 55212 failed to inhibit NO production in cultures from CB1R knockouts. Dibutyryl-cAMP blocked the protective effect of *R*(+)-Win 55212, and this was reversed by the protein kinase A (PKA) inhibitor *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89). Cannabinoids seem to protect neurons against NMDA toxicity at least in part by activation of CB1R and downstream inhibition of PKA signaling and NO generation.

Cannabinoid receptor agonist drugs protect neurons from the cytotoxic effects of excitatory amino acids (Shen and Thayer, 1998) and from pathological processes, such as ischemia (Nagayama et al., 1999), in which excitotoxicity has been implicated. Endogenous cannabinoid (endocannabinoid) signaling is also neuroprotective and may help to promote the survival of injured neural tissue. Examples of endogenous cannabinergic neuroprotection include the ability of endocannabinoids to rescue neurons from hypoxia (Sinor et al., 2000) and trauma (Panikashvili et al., 2001), and the exacerbation of ischemic (Parmentier-Batteur et al., 2002), excitotoxic (Parmentier-Batteur et al., 2002; Marsicano et al., 2003), and traumatic (Panikashvili et al., 2005) neuronal injury observed in CB1R cannabinoid receptor-knockout mice. These observations are consistent with a role for endogenous cannabinoids (Franklin et al., 2003) and their receptors (Jin et al., 2000) as injury-inducible mediators of neuronal adaptation and survival.

CB1R activation mobilizes a large number of signal transduction pathways, but which of these is critical for neuroprotection from excitotoxic disorders is unclear. Because CB1Rs are largely presynaptic (Maejima et al., 2001; Wilson et al., 2001) and can inhibit Ca^{2+} influx through voltage-gated Ca^{2+} channels (Mackie and Hille, 1992), their antiexcitotoxic effects might stem partly from reducing Ca^{2+}-dependent release of excitatory neurotransmitters, such as glutamate. However, the ability of cannabinoids to block the toxicity of exogenous glutamate or its analogs cannot easily be explained by the inhibition of glutamate release and suggests a postsynaptic action downstream of glutamate receptor acti-

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ABBREVIATIONS: CB1R, CB1 cannabinoid receptor; PKA, protein kinase A; dbcAMP, dibutyryl-cyclic adenosine monophosphate (dbcAMP); NMDA, *N*-methyl-D-aspartate; *R*(+)-Win 55212, *R*(+)-[2, 3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-cichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; 7-NI, 7-nitroindazole; L-NAME, *N*-ω-nitro-L-arginine methyl ester; H89, *N*[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; PTX, pertussis toxin; NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; LDH, lactate dehydrogenase; KO, knockout; WT, wild type; ANOVA, analysis of variance; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate.

vation. In this respect, signaling molecules that are affected oppositely by excitotoxins and by cannabinoids are promising candidate mediators of neuroprotection.

Of numerous signal transduction pathways implicated in neuronal excitotoxicity, the generation of NO through the activation of neuronal nitric-oxide synthase (nNOS) is among the best characterized. NO generated by nNOS and by endothelial NOS and inducible NOS (iNOS) regulates the severity of cerebral ischemic injury and also seems to be a downstream effector of cannabinoid effects. Gene-knockout studies have shown that nNOS and iNOS exacerbate neuronal injury from focal cerebral ischemia induced by middle cerebral artery occlusion, whereas endothelial NOS attenuates ischemic injury (Samdani et al., 1997). Pharmacological studies also point to a major role for NO in excitotoxic, hypoxic, and ischemic neuronal injury (Samdani et al., 1997) and perhaps in ischemic tolerance (Gidday et al., 1999).

Both nNOS and iNOS also have been implicated in cannabinoid signaling. In cerebellar granule cells, CB1R activation inhibits the depolarization- and Ca^{2+}-dependent production of NO by nNOS (Hillard et al., 1999). In addition, CB1R and nNOS are coexpressed in neurons in a variety of brain regions, and some but not all of the central nervous system effects of the naturally occurring plant cannabinoid, Δ^9-tetrahydrocannabinol, are lost in nNOS-KO mice (Azad et al., 2001). CB1R activation also inhibits the lipopolysaccharide and interferon-γ-stimulated generation of NO by iNOS in microglial cells (Waksman et al., 1999), apparently by promoting the release of endogenous interleukin-1 receptor antagonist (Molina-Holgado et al., 2003). The NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) potentiates the central hypothermic effect of *R*(+)-Win 55212 in rats (Rawls et al., 2004). The endocannabinoid anandamide stimulates NO synthesis in endothelial cells, and anandamide-induced vasodilation is blocked by L-NAME (Deutsch et al., 1997). Finally, cannabinoids prevent the retinal neurotoxicity of NMDA; this toxicity is both associated with generation of peroxynitrite from NO and reduced by L-NAME (El-Remessy et al., 2003). Together, these findings suggest that the neuroprotective action of cannabinoids in excitotoxic injury might involve the effects of cannabinoids on one or more NOS isoforms.

To investigate the possible role of NO signaling in the antiexcitotoxic effects of cannabinoids on cerebral neurons, we studied NMDA-induced neuronal cell death and NO production in cell cultures and in vivo using wild-type and CB1R-knockout mice. We report that the ability of cannabinoids to protect neurons from excitotoxic injury depends on the inhibition of NOS and PKA.

Materials and Methods

Drugs. *R*(+)-Win 55212 was purchased from Sigma/RBI (Natick, MA), and SR141716A was obtained from the National Institute on Drug Abuse (Rockville, MD). *N*-Methyl-D-aspartate (NMDA), 7-nitroindazole (7-NI), and H89 were purchased from Sigma-Aldrich (St. Louis, MO). Pertussis toxin (PTX) and adenosine 3′,5′-cyclic monophosphate dibutyryl sodium salt (dbcAMP) were obtained from Calbiochem (San Diego, CA).

Mice. CB1 knockout and wild-type littermate mice, bred for at least five generations on a CD1 background, were generously provided by Dr. Catherine Ledent (Ledent et al., 1999) and were used to breed mice for this study. Genotyping was performed on the basis of the protocol used by of Ledent et al. (1999), as described previously (Parmentier-Batteur et al., 2002).

Primary Cortical Cell Culture. Neuron-enriched mouse cerebral cortical cultures were prepared from the brains of embryonic day-16 wild-type CD1 and CB1 knockout mice. Neocortex was triturated, and dissociated cells were plated at five hemicortices per six- or 24-well plastic culture plate in Eagle's minimal essential medium (Earle's salts, supplied glutamine-free) supplemented with 5% horse serum, 5% fetal bovine serum, 21 mM glucose, 26.5 mM bicarbonate,

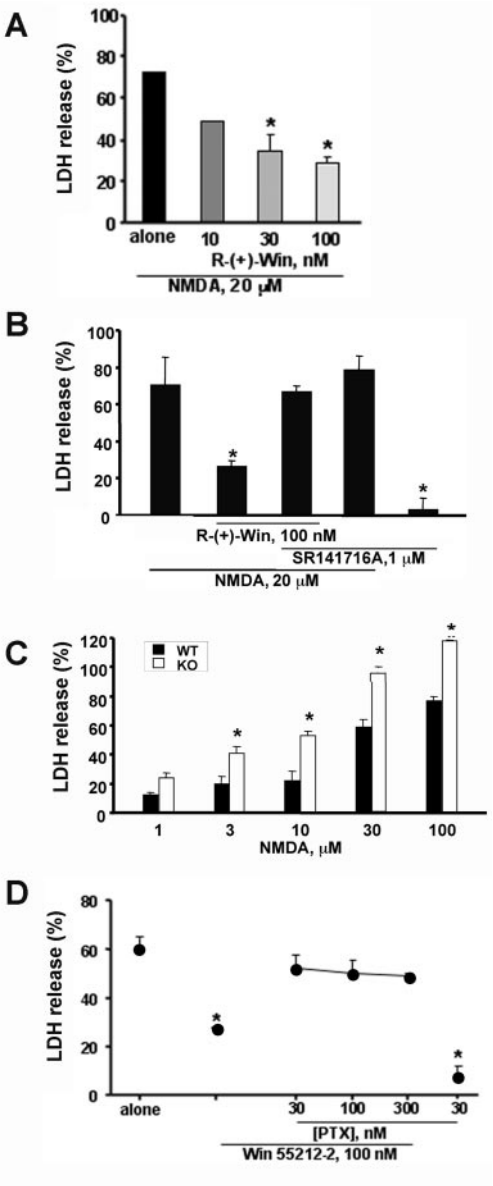

Fig. 1. CB1 receptor-mediated protection of cultured neurons from NMDA-induced neuronal injury. Cortical cell cultures (12–15 days in vitro) were exposed to NMDA for 24 h in the absence and presence of *R*(+)-Win 55212 (A) or *R*(+)-Win 55212 plus or minus SR141716A (B). Cell death was measured by LDH efflux into the medium. Data shown are means ± S.E.M. from 20 wells per condition. *, $p < 0.05$ compared with NMDA alone (ANOVA and Student-Newman-Keuls tests). C, cortical cell cultures from wild-type (WT) and CB1R-KO mice were exposed to the indicated concentrations of NMDA for 24 h. Data shown are means ± S.E.M. from 16 wells per condition. *, $p < 0.05$ compared with WT (ANOVA and Student-Newman-Keuls tests). D, cortical cell cultures were exposed to NMDA for 24 h, in the absence (alone) and presence of *R*(+)-Win 55212, *R*(+)-Win 55212 plus PTX, or PTX alone. Data shown are means ± S.E.M. from 16 wells per condition. *, $p < 0.05$ compared with NMDA alone (ANOVA and Student-Newman-Keuls tests).

and 2 mM L-glutamine. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator and, beginning 2 days after plating, were given fresh medium lacking fetal serum twice weekly. Cytosine arabinoside (10 μM) was added for days 5 to 7 in vitro.

Measurement of Cell Death. Between days 12 and 14 in vitro, cultures were rinsed with serum-free minimal essential medium and treated for 24 h with 20 μM NMDA, with or without other drugs. Cell death was quantified by measuring lactate dehydrogenase (LDH) release into the bathing medium over 24 h and was expressed as a percentage of cell death induced by a maximally cytotoxic concentration (500 μM) of NMDA: $(LDH - LDH_{control})/(LDH_{NMDA} - LDH_{control}) \times 100\%$.

Intracerebral Injection of NMDA. Mice were anesthetized with 1.5% isoflurane in 70% N_2O/30% O_2. NMDA was administered in vivo as described previously (Parmentier-Batteur et al., 2002), by injection of 10 nmol NMDA in 0.5 μl of sterile phosphate-buffered saline into the parietal cortex at a site 1.5 mm caudal to bregma, 3.0 mm from the midline, and 0.8 mm below the dural surface. After 24 h, 30-μm coronal brain sections were stained with hematoxylin to delineate the resulting lesion.

NOS Activity Assay. Cerebral cortex was dissected on ice 1 h after cerebral injection of NMDA. NOS activity was measured using a commercial colorimetric assay kit (Cayman Chemical, Ann Arbor, MI), in which the conversion of nitrate to nitrite by nitrate reductase is assayed using the Griess reagent. Absorbance was read at 570/620 nm by using a Cytofluor series 4000 multiwell plate reader (Applied Biosystems, Foster City, CA).

Treatment with 7-NI. 7-NI (50 mg/kg i.p.) was dissolved in dimethyl sulfoxide/1,2-propanediol/distilled water (1:3:6) and administered 30 min before and 2 h after intracerebral injection of NMDA. Control animals received the same volume of vehicle at the same times. After 24 h, 30-μm coronal brain sections were stained with hematoxylin to delineate the resulting lesion, and lesion area and volume were determined as described previously (Parmentier-Batteur et al., 2002).

Detection of Nitric Oxide Generation. Cultures were loaded with 1 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate; Invitrogen, Carlsbad, CA) in HEPES-buffered control salt solution, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES, and 10 mM NaOH. Cultures were incubated for 20 min at 37°C and washed three times with HEPES-buffered control salt solution. The fluorescence signal was observed at excitation of 495 nm and emission of 515 nm with a Nikon E800 fluorescence microscope (Nikon, Tokyo, Japan).

Immunocytochemistry. Cultures were fixed in 4% paraformaldehyde for 30 min, incubated in 5% horse serum for 1 h, and immunolabeled with mouse monoclonal anti-nNOS (1:500; Transduction Laboratories, Lexington, KY) and rabbit polyclonal anti-CB1R (1:500; Calbiochem, San Diego, CA) at 4°C overnight. Cultures were washed with phosphate-buffered saline and reacted with fluorescein isothiocyanate-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) for 1 h. The fluorescence signals were detected at excitation of 470 nm and emission of 505 nm.

Data Analysis. Data were expressed as mean ± S.E.M. ANOVA and Student-Newman-Keuls test (multiple comparisons) or Student's t test (single comparisons) was used for statistical analysis, with $p < 0.05$ considered significant.

Results

Exposure for 24 h to 20 μM NMDA caused the death of ~70% of cells in neuronally enriched murine cortical cultures, as measured by LDH release (Fig. 1A). In the presence of the cannabinoid agonist $R(+)$-Win 22512 (100 nM), NMDA toxicity was reduced by ~65% (Fig. 1B). Protection was abolished by the CB1R cannabinoid receptor antagonist SR141716A (1 μM), consistent with a CB1R-mediated effect. This was confirmed by comparing NMDA toxicity in cortical neuron cultures from wild-type and CB1R-knockout mice. In both cases, NMDA produced concentration-dependent neurotoxicity, but toxicity was greater in the knockouts (Fig. 1C).

Some actions of cannabinoids are dependent on PTX-sensitive G-proteins. To evaluate whether this applies to protection from NMDA toxicity as well, PTX (which disassociates G_i- from G_i-coupled receptors) was added to some cultures for the duration of NMDA exposure. As reported previously (Kim et al., 2005), PTX alone had no effect on LDH release at the concentrations studied. However, it reversed protection from NMDA toxicity by $R(+)$-Win 55212 (Fig. 1D).

NO has been implicated in both excitotoxicity and cannabinoid signaling. Therefore, NMDA toxicity in our cultures was reduced by two NOS inhibitors, 7-NI and L-NAME (Fig.

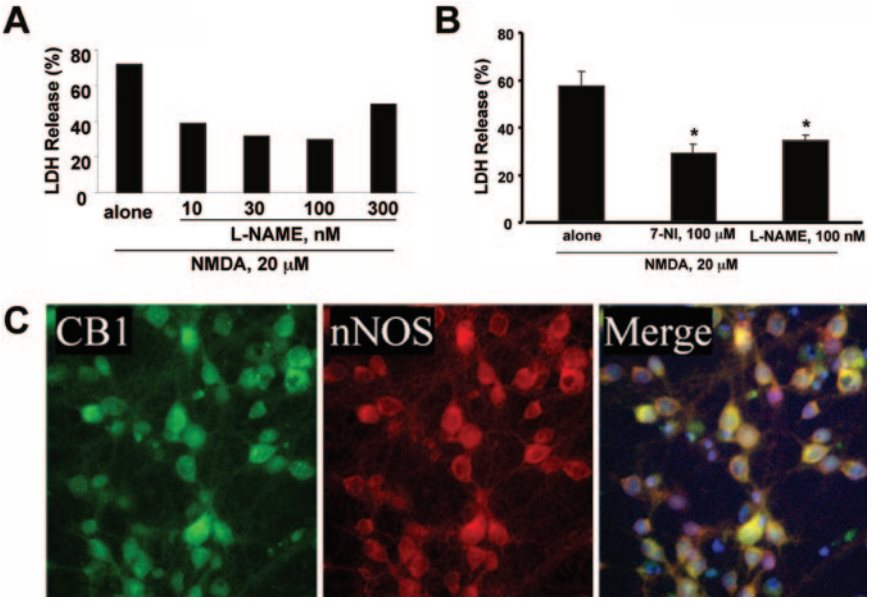

Fig. 2. Involvement of NOS in NMDA-induced neurotoxicity. Cortical cell cultures were exposed to NMDA for 24 h in the absence and presence of various concentrations of L-NAME (A) or maximally effective concentrations of 7-NI or L-NAME (B). Cell death was measured by LDH efflux into the medium. Data shown are means ± S.E.M. from 16 wells per condition. *, $p < 0.05$ compared with NMDA alone (ANOVA and Student-Newman-Keuls tests). C, fluorescence photomicrograph of cortical cell cultures immunolabeled with an anti-CB1 antibody (green) and an anti-nNOS antibody (red) shows colocalization of CB1 and nNOS in the same cells; nuclei are counterstained with 4,6-diamidino-2-phenylindole.

2, A and B). Moreover, the presence of CB1R on nNOS-expressing cells (Fig. 2C) suggested that CB1R-mediated protection from NMDA toxicity might involve the induction of neuronal NO synthesis. To explore this possibility, we first examined the effect of CB1R deletion on NMDA-induced cerebral cortical NOS activity in vivo, which was increased by ~50% in CB1R-knockout compared with wild-type mice (Fig. 3A). We reported previously that the size of lesions produced by intracortical injection of NMDA is increased in CB1R-knockout mice (Parmentier-Batteur et al., 2002). If the increased NMDA toxicity observed in CB1R-knockout mice is related to increased NO synthesis, then suppression of NO synthesis should restore toxicity toward levels observed in wild-type mice. To test this prediction, we administered the NOS inhibitor 7-NI, or vehicle, to CB1R-knockout mice, 30 min before and 120 min after intracortical NMDA. Lesion volume was reduced by ~60% in 7-NI-treated mice (Fig. 3, B and C), consistent with the involvement of NO signaling in

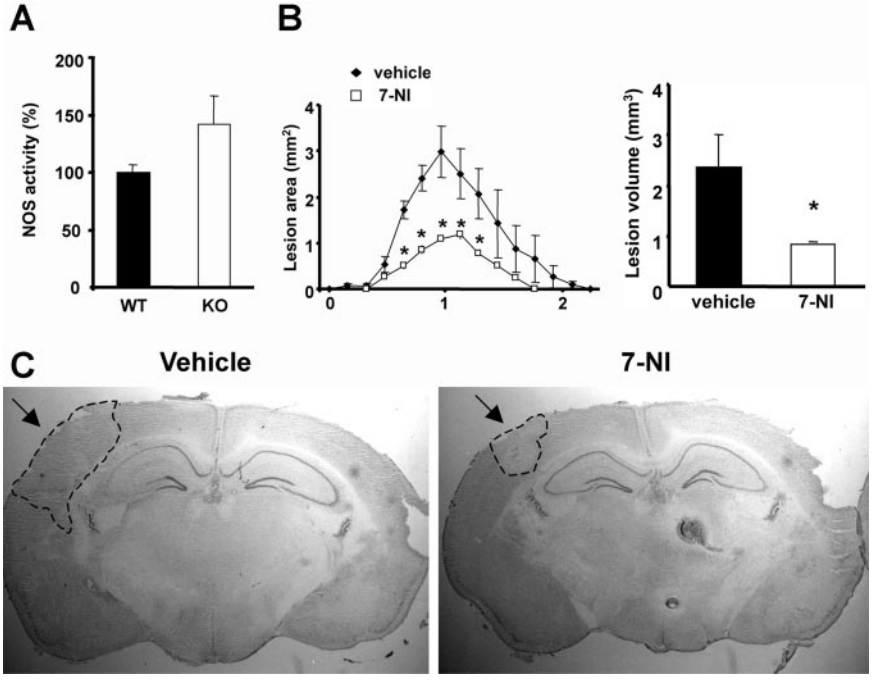

Fig. 3. NOS enhances NMDA injury in CB1R knockout mice. A, NOS activity was assayed in cerebral cortex of WT and KO mice 30 min after NMDA injection. Data shown are means ± S.E.M. from four mice per condition. *, $p < 0.05$ compared with WT mice (ANOVA and Student-Newman-Keuls tests). B, NMDA (10 nmol) was injected into the parietal cortex of KO mice. The vehicle or 7-NI was injected intraperitoneally 30 min before and 2 h after NMDA injection; the area of the resulting hematoxylin-unstained lesion was measured at multiple coronal levels (left), and the volume (right) was calculated therefrom. Data shown are means ± S.E.M. from four KO mice per condition. *, $p < 0.05$ compared with vehicle-injected KO mice (left, two-tailed Student's t test; right, Student's t test). C, representative KO brains treated with vehicle or 7-NI as described under *Materials and Methods* show NMDA-induced lesions of different size (outlined areas).

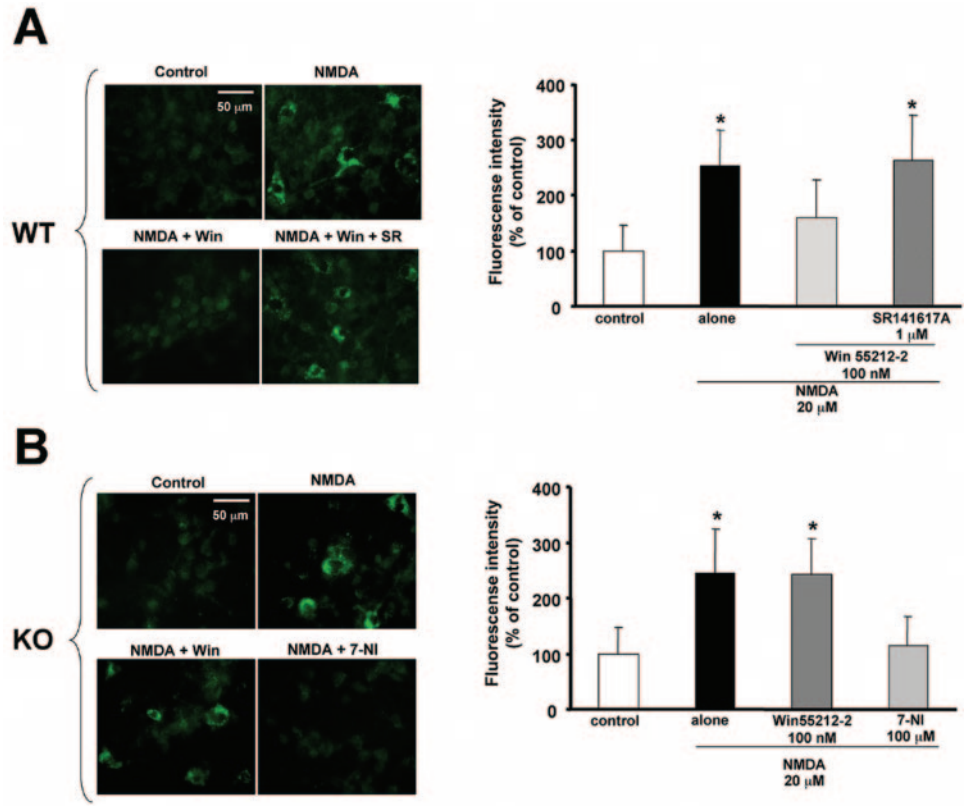

Fig. 4. CB1R-mediated production of NO. Fluorescence photomicrograph (left) and quantitation (right) of NO produced in cortical neurons loaded with the nitric oxide-sensitive dye DAF-FM at 6 h after exposure to a sham operation (control), 20 mM NMDA, or 20 mM NMDA plus 100 nM $R(+)$-Win 55212 with or without 1 mM SR141617A in cortical cell cultures of WT (A), and a sham operation (control), 20 mM NMDA, or 20 mM NMDA plus 100 nM $R(+)$-Win 55212 or 100 mM 7-NI in cortical cell cultures of CB1R-knockout mice (B, KO). *, significant difference from the control at $p < 0.05$ using analysis of variance and Student-Newman-Keuls test.

the enhanced NMDA toxicity that occurs after CB1R deletion.

To determine how CB1R activation modifies NMDA-stimulated NO production, we first measured fluorescence intensity in wild-type cortical cultures loaded with the NO-indicator dye DAF-FM (Kojima et al., 2001). NMDA (20 μM) increased fluorescence by ~160%; *R*(+)-Win 55212 blocked the effect of NMDA, which was restored in the presence of SR141716A (Fig. 4A). We next evaluated how NMDA influences NO production in cortical cultures from CB1R-knockout mice. In these cultures, NMDA-stimulated production of NO was unaffected by *R*(+)-Win 55212, consistent with the absence of CB1R, but was prevented by 7-NI (Fig. 4B). Therefore, CB1R activation seems to act upstream of NO synthesis to inhibit NMDA toxicity.

In a previous study (Kim et al., 2005), we found that attenuation of $FeCl_2$-induced oxidative neuronal injury by cannabinoids occurs via the inhibition of PKA. To investigate whether a similar mechanism is involved in cannabinoid protection from excitotoxicity, some cultures were treated with NMDA in the presence or absence of *R*(+)-Win 55212 and with or without dbcAMP, which activates PKA. The protective effect of *R*(+)-Win 55212 against NMDA toxicity was abolished by dbcAMP (Fig. 5A), as was inhibition by

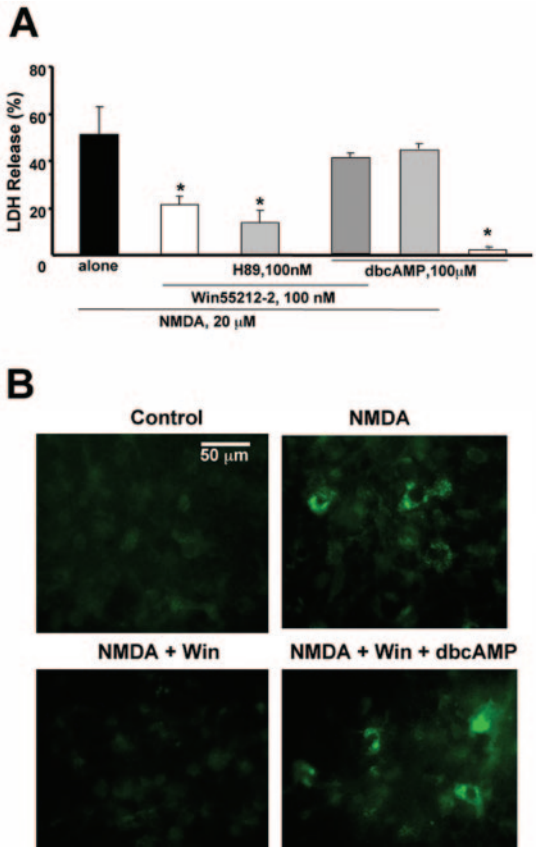

Fig. 5. Involvement of PKA in the neuroprotective effect of CB1R. A, cortical cell cultures were exposed to NMDA for 24 h in the absence and presence of *R*(+)-Win 55212 or *R*(+)-Win 55212 plus dbcAMP or H89, or to dbcAMP alone. Cell death was measured by LDH efflux into the medium. Data shown are means ± S.E.M. from 16 wells per condition. *, $p < 0.05$ compared with NMDA alone (ANOVA and Student-Newman-Keuls tests). B, fluorescence photomicrograph of cortical neurons loaded with the nitric oxide-sensitive dye DAF-FM after exposure to a sham operation (control), 20 mM NMDA, or 20 mM NMDA plus 100 nM *R*(+)-Win 55212 with or without 100 mM dbcAMP for 6 h.

R(+)-Win 55212 of NMDA-induced NO synthesis (Fig. 5B). Finally, the PKA inhibitor H89 inhibited the effect of dbcAMP.

Discussion

The main finding reported here is that cannabinoids protect neurons from excitotoxic injury by a mechanism that involves the activation of CB1R and inhibition of NOS and PKA. This was demonstrated in both cultured cerebrocortical neurons in vitro and the brains of mice in vivo and was confirmed by studies with CB1R-knockout mice.

Endogenous or exogenous cannabinoids have shown neuroprotective effects in some studies (Shen and Thayer, 1998; Nagayama et al., 1999; Sinor et al., 2000; Panikashvili et al., 2001, 2005; Parmentier-Batteur et al., 2002; Marsicano et al., 2003) but not all (Chan et al., 1998; Campbell, 2001). Cannabinoid-induced neuroprotection has been observed in animal models of stroke (Nagayama et al., 1999), head trauma (Panikashvili et al., 2001), epilepsy (Marsicano et al., 2003), brain tumor (Galve-Roperh et al., 2000), and multiple sclerosis (Pryce et al., 2003). In addition to directly salvaging neurons affected in these disorders, cannabinoids also have anti-inflammatory effects (Jeon et al., 1996) and promote the birth of new neurons (neurogenesis) in the adult brain (Jin et al., 2004), either of which may contribute to improving neurological outcome. Like the neuroprotective effects of cannabinoids observed in the present study, their anti-inflammatory action involves NO signaling (Waksman et al., 1999). It is unclear whether enhancement of neurogenesis by cannabinoids is similarly dependent on NO, although NO has been implicated in adult neurogenesis under some circumstances (Packer et al., 2003). Thus, interaction between the cannabinoid and NO signaling pathways may be a common feature of a variety of cannabinoid actions, which have the net result of mitigating neuronal injury or stimulating repair and recovery.

PKA has been implicated in the effects of cannabinoids, although not in a consistent manner. The PKA activator cAMP potentiated the protective effect of cannabinoids against glutamate toxicity in cultured rat neurons (Hampson and Grimaldi, 2001). In contrast, PKA activation reduced the inhibitory effect of *R*(+)-Win 55212 on presynaptic glutamate release in corticostriatal brain slices (Huang et al., 2002). We reported the involvement of PKA in cannabinoid protection of cultured cortical neurons from oxidative injury (Kim et al., 2005). Dibutyryl cAMP blocked the protective action of *R*(+)-Win 55212 on $FeCl_2$-, H_2O_2-, or buthionine sulfoximine-induced cell death, and the effect of dbcAMP was inhibited by the PKA inhibitor H89. This resembles findings regarding NMDA toxicity in the present study and suggests that the role of PKA inhibition, like that of NOS inhibition, may be important across a range of cannabinoid-sensitive neuropathological processes. CB1R activation regulates adenylate cyclase and, in turn, PKA activity, but CB1R is also a substrate for phosphorylation by PKA (Huang et al., 2002). Therefore, the precise site or sites at which dbcAMP and H89 interact with cannabinoid signaling in our system are uncertain. Additional mechanisms triggered by cannabinoids may also contribution to neuroprotection. For example, signaling molecules that regulate cell survival and which are influenced by cannabinoids include protein kinase B (Gomez del

Pulgar et al., 2000) and nuclear factor-κB (Panikashvili et al., 2005).

One reason for trying to unravel the signal transduction mechanisms associated with cannabinoid neuroprotection is that cannabinoids have pleiotropic effects, some of which may tend to result in adverse outcomes. For example, systemic hypotension produced by cannabinoids may have a detrimental effect in disorders, like stroke and head trauma, in which cerebral perfusion is already impaired. Conversely, some physiological effects of cannabinoids that are ostensibly unrelated to neuroprotection, such as induction of hypothermia, may confer a broad benefit. By identifying the signaling pathways responsible for cannabinoid effects in animal models of disease and their human counterparts, it may be possible to design more specific and therefore more efficacious cannabinoid-based therapies.

References

Azad SC, Marsicano G, Eberlein I, Putzke J, Zieglgansberger W, Spanagel R, and Lutz B (2001) Differential role of the nitric oxide pathway on Δ^9-THC-induced central nervous system effects in the mouse. *Eur J Neurosci* **13:**561–568.

Campbell VA (2001) Tetrahydrocannabinol-induced apoptosis of cultured cortical neurones is associated with cytochrome c release and caspase-3 activation. *Neuropharmacology* **40:**702–709.

Chan GC-K, Hinds TR, Impey S, and Storm DR (1998) Hippocampal neurotoxicity of Δ9-tetrahydrocannabinol. *J Neurosci* **18:**5322–5332.

Deutsch DG, Goligorsky MS, Schmid PC, Krebsbach RJ, Schmid HH, Das SK, Dey SK, Arreaza G, Thorup C, Stefano G, et al. (1997) Production and physiological actions of anandamide in the vasculature of the rat kidney. *J Clin Investig* **100:**1538–1546.

El-Remessy AB, Khalil IE, Matragoon S, Abou-Mohamed G, Tsai NJ, Roon P, Caldwell RB, Caldwell RW, Green K, and Liou GI (2003) Neuroprotective effect of (−)delta9-tetrahydrocannabinol and cannabidiol in *N*-methyl-D-aspartate-induced retinal neurotoxicity: involvement of peroxynitrite. *Am J Pathol* **163:**1997–2008.

Franklin A, Parmentier-Batteur S, Walter L, Greenberg DA, and Stella N (2003) Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. *J Neurosci* **23:**7767–7775.

Galve-Roperh I, Sanchez C, Cortes ML, del Pulgar TG, Izquierdo M, and Guzman M (2000) Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat Med* **6:**313–319.

Gidday JM, Shah AR, Maceren RG, Wang Q, Pelligrino DA, Holtzman DM, and Park TS (1999) Nitric oxide mediates cerebral ischemic tolerance in a neonatal rat model of hypoxic preconditioning. *J Cereb Blood Flow Metab* **19:**331–340.

Gomez del Pulgar T, Velasco G, and Guzman M (2000) The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. *Biochem J* **347 (Pt 2):**369–373.

Hampson AJ and Grimaldi M (2001) Cannabinoid receptor activation and elevated cyclic AMP reduce glutamate neurotoxicity. *Eur J Neurosci* **13:**1529–1536.

Hillard CJ, Muthian S, and Kearn CS (1999) Effects of CB_1 cannabinoid receptor activation on cerebellar granule cell nitric oxide synthase activity. *FEBS Lett* **459:**277–281.

Huang CC, Chen YL, Lo SW, and Hsu KS (2002) Activation of cAMP-dependent protein kinase suppresses the presynaptic cannabinoid inhibition of glutamatergic transmission at corticostriatal synapses. *Mol Pharmacol* **61:**578–585.

Jeon YJ, Yang KH, Pulaski JT, and Kaminski NE (1996) Attenuation of inducible nitric oxide synthase gene expression by Δ9-tetrahydrocannabinol is mediated through the inhibition of nuclear factor-κB/Rel activation. *Mol Pharmacol* **50:**334–341.

Jin K, Xie L, Kim SH, Parmentier-Batteur S, Sun Y, Mao XO, Childs J, and Greenberg DA (2004) Defective adult neurogenesis in CB1 cannabinoid receptor knockout mice. *Mol Pharmacol* **66:**204–208.

Jin KL, Mao XO, Goldsmith PC, and Greenberg DA (2000) CB1 cannabinoid receptor induction in experimental stroke. *Ann Neurol* **48:**257–261.

Kim SH, Won SJ, Mao XO, Jin K, and Greenberg DA (2005) Involvement of protein kinase A in cannabinoid receptor-mediated protection from oxidative neuronal injury. *J Pharmacol Exp Ther* **313:**88–94.

Kojima H, Hirata M, Kudo Y, Kikuchi K, and Nagano T (2001) Visualization of oxygen-concentration-dependent production of nitric oxide in rat hippocampal slices during aglycemia. *J Neurochem* **76:**1404–1410.

Ledent C, Valverde O, Cossu G, Petitet F, Aubert J-F, Beslot F, Böhme GA, Imperato A, Pedrazzini T, Roques BP, et al. (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science (Wash DC)* **283:**401–404.

Mackie K and Hille B (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc Natl Acad Sci USA* **89:**3825–3829.

Maejima T, Hashimoto K, Yoshida T, Aiba A, and Kano M (2001) Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. *Neuron* **31:**463–475.

Marsicano G, Goodenough S, Monory K, Hermann H, Eder M, Cannich A, Azad SC, Cascio MG, Gutierrez SO, van der Stelt M, et al. (2003) CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science (Wash DC)* **302:**84–88.

Molina-Holgado F, Pinteaux E, Moore JD, Molina-Holgado E, Guaza C, Gibson RM, and Rothwell NJ (2003) Endogenous interleukin-1 receptor antagonist mediates anti-inflammatory and neuroprotective actions of cannabinoids in neurons and glia. *J Neurosci* **23:**6470–6474.

Nagayama T, Sinor AD, Simon RP, Chen J, Graham SH, Jin K, and Greenberg DA (1999) Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J Neurosci* **19:**2987–2995.

Packer MA, Stasiv Y, Benraiss A, Chmielnicki E, Grinberg A, Westphal H, Goldman SA, and Enikolopov G (2003) Nitric oxide negatively regulates mammalian adult neurogenesis. *Proc Natl Acad Sci USA* **100:**9566–9571.

Panikashvili D, Mechoulam R, Beni SM, Alexandrovich A, and Shohami E (2005) CB1 cannabinoid receptors are involved in neuroprotection via NF-kappa B inhibition. *J Cereb Blood Flow Metab* **25:**477–484.

Panikashvili D, Simeonidou C, Ben-Shabat S, Hanus L, Breuer A, Mechoulam R, and Shohami E (2001) An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature (Lond)* **413:**527–531.

Parmentier-Batteur S, Jin K, Mao XO, Xie L, and Greenberg DA (2002) Increased severity of stroke in CB1 cannabinoid receptor knock-out mice. *J Neurosci* **22:**9771–9775.

Pryce G, Ahmed Z, Hankey DJ, Jackson SJ, Croxford JL, Pocock JM, Ledent C, Petzold A, Thompson AJ, Giovannoni G, et al. (2003) Cannabinoids inhibit neurodegeneration in models of multiple sclerosis. *Brain* **126 (Pt 10):**2191–2202.

Rawls SM, Tallarida RJ, Gray AM, Geller EB, and Adler MW (2004) L-NAME (N^{ω}-nitro-L-arginine methyl ester), a nitric-oxide synthase inhibitor and WIN 55212–2 [4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenylcarbonyl)-6*H*-pyrrolo[3,2,1*ij*]quinolin-6-one], a cannabinoid agonist, interact to evoke synergistic hypothermia. *J Pharmacol Exp Ther* **308:**780–786.

Samdani AF, Dawson TM, and Dawson VL (1997) Nitric oxide synthase in models of focal ischemia. *Stroke* **28:**1283–1288.

Shen M and Thayer SA (1998) Cannabinoid receptor agonists protect cultured rat hippocampal neurons from excitotoxicity. *Mol Pharmacol* **54:**459–462.

Sinor AD, Irvin SM, and Greenberg DA (2000) Endocannabinoids protect cerebral cortical neurons from in vitro ischemia in rats. *Neurosci Lett* **278:**157–160.

Waksman Y, Olson JM, Carlisle SJ, and Cabral GA (1999) The central cannabinoid receptor (CB1) mediates inhibition of nitric oxide production by rat microglial cells. *J Pharmacol Exp Ther* **288:**1357–1366.

Wilson RI, Kunos G, and Nicoll RA (2001) Presynaptic specificity of endocannabinoid signaling in the hippocampus. *Neuron* **31:**453–462.

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