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Molecular mechanisms of cannabinoid protection from neuronal excitotoxicity

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Running title: Cannabinoids and excitotoxicity

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Nonstandard abbreviations: CB1 cannabinoid receptor (CB1R); protein kinase A (PKA); dibutyryl-cyclic adenosine monophosphate (dbcAMP); 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); *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-cichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A); 7-nitroindazole (7-NI); N-ω-nitro-L-arginine methyl ester (L-NAME); N[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89); pertussis toxin (PTX); nitric oxide synthase (NOS); minimal essential medium (MEM); lactate dehydrogenase (LDH); Hepes-buffered control salt solution (HCSS)**;** fluorescein isothiocyanate (FITC)

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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 CB1R antagonist N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-cichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A). Cultures from CB1R-knockout mice were more sensitive to NMDA toxicity than 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-nitro-L-arginine methyl ester (L-NAME) 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 (DAF-FM) diacetate, and this effect was reversed by SR141716A. In contrast, R(+)-Win 55212 failed to inhibit NO production in cultures from CB1R knockouts. Dibutyryl-cyclic adenosine monophosphate (dbcAMP) blocked the protective effect of R(+)-Win 55212, and this was reversed by the protein kinase A inhibitor N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89). Cannabinoids appear to protect neurons against NMDA toxicity at least partly by activation of CB1R and downstream inhibition of PKA signaling and NO generation.

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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 (Marsicano et al., 2003; Parmentier-Batteur et al., 2002) 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 are critical for neuroprotection from excitotoxic disorders is unclear. Because CB1R 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 anti-excitotoxic 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 inhibition of glutamate release, and suggests a postsynaptic action downstream of glutamate receptor activation. 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 activation of neuronal nitric oxide synthase (nNOS) is among the best characterized. NO generated by nNOS, as well as by endothelial NOS (eNOS) and inducible NOS (iNOS), regulates the severity of cerebral ischemic injury and also appears to be a downstream effector of cannabinoid effects. Gene-knockout studies have shown that

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nNOS and iNOS exacerbate neuronal injury from focal cerebral ischemia induced by middle cerebral artery occlusion, whereas eNOS 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 also in ischemic tolerance (Gidday et al., 1999).

Both nNOS and iNOS have also 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 co-expressed in neurons in a variety of brain regions, and some but not all of the CNS effects of the naturally occurring plant cannabinoid, Δ^9-tetrahydrocannabinol (THC), 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 (IL-1ra) (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 (AEA) stimulates NO synthesis in endothelial cells, and AEA-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 effects of cannabinoids on one or more NOS isoforms.

To investigate the possible role of NO signaling in the anti-excitotoxic 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 inhibition of NOS and of PKA.

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Methods

Drugs. 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) was purchased from Research Biochemicals (Natick, MA) and *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-cichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A) was obtained from the National Institute on Drug Abuse (Rockville, MD). N-Methyl-D-aspartate (NMDA), 7-nitroindazole (7-NI) and N[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89) were purchased from Sigma (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 knock-out 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 used to breed mice for this study. Genotyping was performed based on the protocol 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 day E16 wild-type CD1 and CB1 knock-out mice. Neocortex was triturated and dissociated cells were plated at five hemicortices per 6- or 24-well plastic culture plate in Eagle's minimal essential medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% horse serum, 5% fetal bovine serum (FBS), 21 mM glucose, 26.5 mM bicarbonate, and 2 mM L-glutamine. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator and, beginning 2 d after plating, were given fresh medium lacking fetal serum twice weekly. Cytosine arabinoside (10 μM) was added for days 5-7 *in vitro*.

Measurement of cell death. Between days 12 and 14 *in vitro*, cultures were rinsed with serum-free MEM and treated for 24 h with 20 μM NMDA, with or without other drugs.

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Cell death was quantified by measuring lactate dehydrogenase (LDH) release into the bathing medium over 24 h and expressed as a percentage of cell death induced by a maximally cytotoxic concentration (500 μM) of NMDA: $(LDH - LDH_{control}) / (LDH_{NMDA, 500\ \mu M} - LDH_{control}) \times 100\%$.

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 (Parmentier-Batteur et al., 2002) , by injection of 10 nmol of NMDA in 0.5 μl of sterile phosphate buffered saline (PBS) 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 hr 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-nitroindazole (7-NI). 7-NI (50 mg/kg, i.p.) was dissolved in DMSO: 1,2-propanediol: distilled water::1: 3: 6 and administered 30 min before and 2 hr after intracerebral injection of NMDA. Control animals received the same volume of vehicle at the same times. After 24 hr, 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; Molecular Probes, Eugene, OR) in Hepes-buffered control salt solution (HCSS, pH 7.4) containing (in mM):

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120 NaCl, 5 KCl, 1.6 $MgCl_2$, 2.3 $CaCl_2$, 15 glucose, 20 Hepes, and 10 NaOH. Cultures were incubated for 20 min at 37°C and washed three times with HCSS. The fluorescence signal was observed at excitation = 495 nm and emission = 515 nm with a Nikon E800 fluorescence microscope.

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, La Jolla, CA) at 4°C overnight. Cultures were washed with PBS and reacted with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG (1:200; Vector Laboratories Inc., Burlingame, CA) for 1 h. The fluorescence signals were detected at excitation = 470 nm and emission = 505 nm.

Data analysis. Data were expressed as mean ± SEM. 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 hr to 20 μM NMDA caused death of ~70% of cells in neuronally enriched murine cortical cultures, as measured by LDH release (**Figure 1A**). In the presence of the cannabinoid agonist R(+)-Win 22512 (100 nM), NMDA toxicity was reduced by ~65% (**Figure 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 (**Figure 1C**).

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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 (**Figure 1D**).

NO has been implicated in both excitotoxicity and cannabinoid signaling. Accordingly, NMDA toxicity in our cultures was reduced by two NOS inhibitors, 7-NI and L-NAME (**Figure 2A-B**). Moreover, the presence of CB1R on nNOS-expressing cells (**Figure 2C**) suggested that CB1R-mediated protection from NMDA toxicity might involve 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 to wild-type mice (**Figure 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 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 (**Figure 3B-C**), consistent with the involvement of NO signaling in 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 **(Figure 4A)**. We next evaluated how NMDA influences NO

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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 **(Figure 4B)**. Therefore, CB1R activation appears 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 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 **(Figure 5A)**, as was inhibition by R(+)-Win 55212 of NMDA-induced NO synthesis **(Figure 5B)**. Finally, the effect of dbcAMP was inhibited by the PKA inhibitor H-89.

Discussion

The main finding reported here is that cannabinoids protect neurons from excitotoxic injury by a mechanism that involves 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 confirmed by studies with CB1R-knockout mice.

Endogenous or exogenous cannabinoids have shown neuroprotective effects in some studies (Marsicano et al., 2003; Nagayama et al., 1999; Panikashvili et al., 2001, 2005; Parmentier-Batteur et al., 2002; Shen and Thayer, 1998; Sinor et al., 2000), but not all (Campbell, 2001; Chan et al., 1998). 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

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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 if 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 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 H-89. 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). Consequently, the precise site or sites at which dbcAMP and H-89 interact with cannabinoid signaling in our system is 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).

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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 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.

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Figure legends

Figure 1. CB1 receptor-mediated protection of cultured neurons from NMDA-induced neuronal injury. Cortical cell cultures (DIV 12–15) were exposed to NMDA for 24 h, in the absence and presence of R(+)-Win 55212 (A) or R(+)-Win 55212 plus or minus SR141617A (B). Cell death was measured by LDH efflux into the medium. Data shown are means ± SEM from 20 wells per condition. * $P < 0.05$ compared to NMDA alone (ANOVA and Student-Newman-Keuls tests). (C) Cortical cell cultures from wild-type (WT) and CB1R-knockout (KO) mice were exposed to the indicated concentrations of NMDA for 24 h. Data shown are means ± SEM from 16 wells per condition. * $P < 0.05$ compared to WT (ANOVA and Student-Newman-Keuls tests). (D) Cortical cell cultures were exposed to NMDA for 24 h, in the absence and presence of R(+)-Win 55212, R(+)-Win 55212 plus pertussis toxin (PTX), or PTX alone. Data shown are means ± SEM from 16 wells per condition. * $P < 0.05$ compared to NMDA alone (ANOVA and Student-Newman-Keuls tests).

Figure 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 ± SEM from 16 wells per condition. * $P < 0.05$ compared to 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 DAPI.

Figure 3. NOS enhances NMDA injury in CB1R knockout mice. (A) NOS activity was assayed in cerebral cortex of WT and KO mice 30 minutes after NMDA injection. Data

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shown are means ± SEM from 4 mice per condition. * P < 0.05 compared to 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 i.p. 30 minutes before and 2 hrs 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 ± SEM from 4 KO mice per condition. * P < 0.05 compared to vehicle-injected control of 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 in method show NMDA-induced lesions of different size (outlined areas).

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Figure 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 hrs after exposure to a sham operation (control), 20 □M NMDA, or 20 □M NMDA plus 100 nM R(+)-Win 55212 with or without 1 □M SR141617A in cortical cell cultures of wild-type (A, WT), and a sham operation (control), 20 □M NMDA, or 20 □M NMDA plus 100 nM R(+)-Win 55212 or 100 □M 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.

Figure 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 H-89, or to dbcAMP alone. Cell death was measured by LDH efflux into the medium. Data shown are means ± SEM from 16 wells per condition. * P < 0.05 compared to NMDA alone (ANOVA and Student-Newman-Keuls tests). (B) Fluorescence photomicrograph of cortical neurons loaded with the nitric oxide-sensitive dye

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DAF-FM after exposure to a sham operation (control), 20 □M NMDA, or 20 □M NMDA plus 100 nM R(+)-Win 55212 with or without 100 □M dbcAMP for 6 hrs.

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FIGURE 1

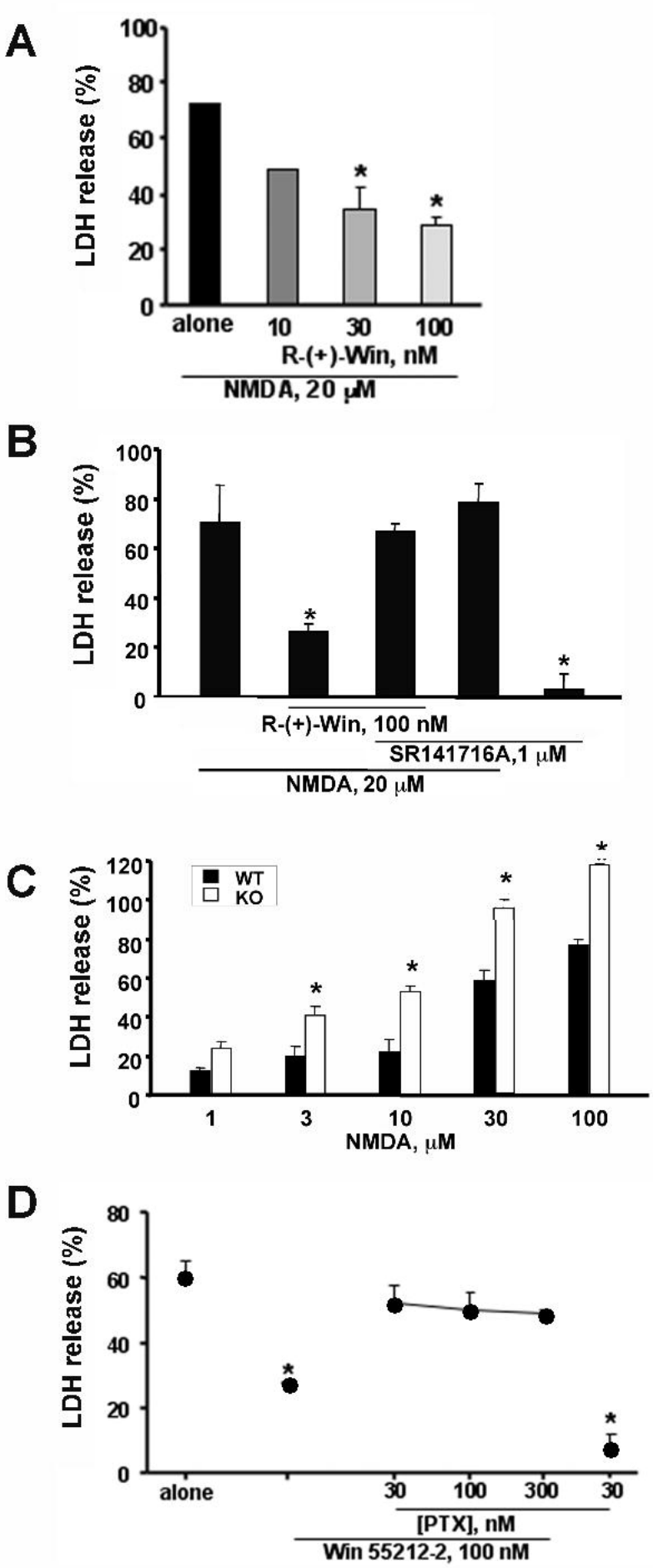

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FIGURE 2

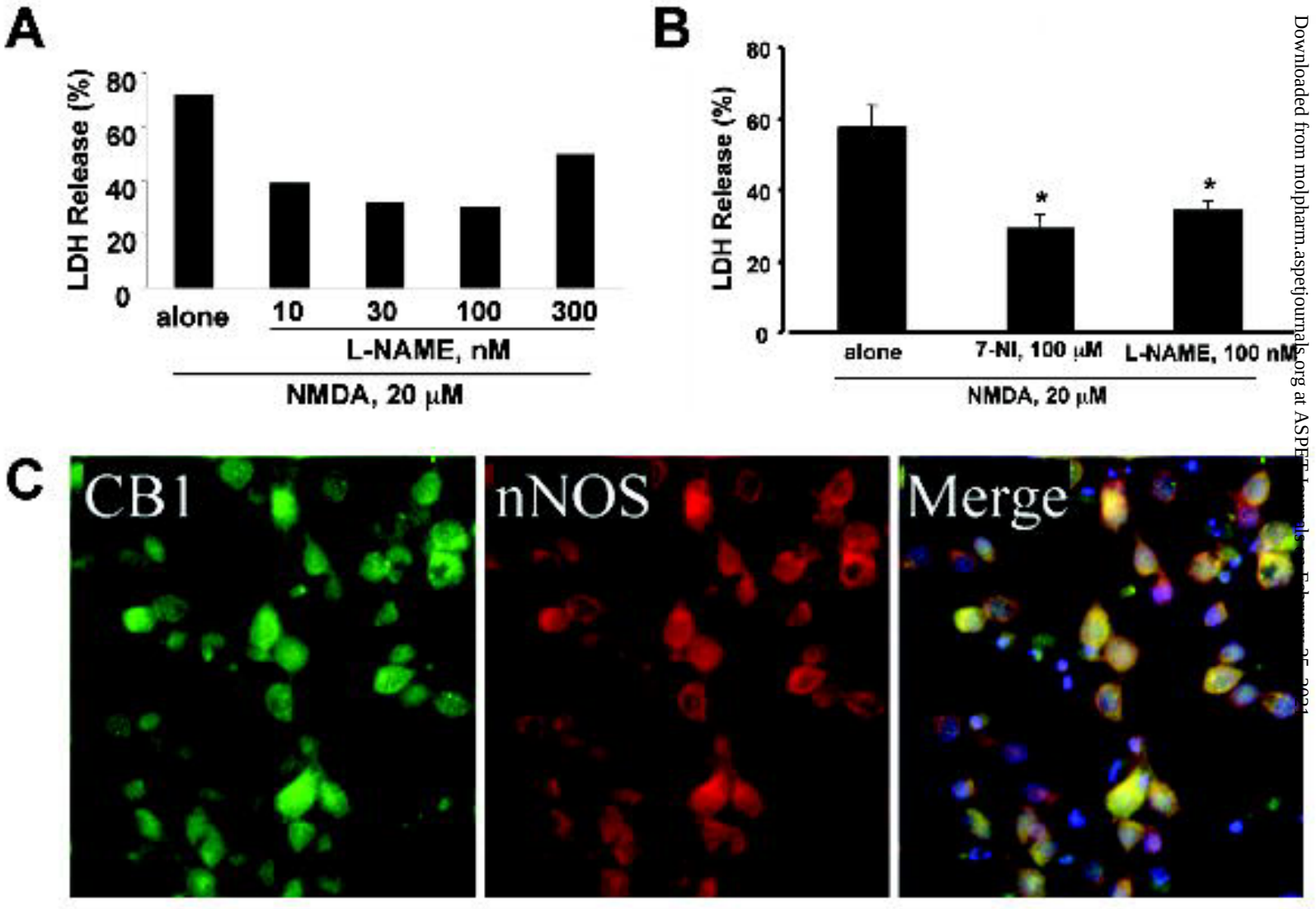

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FIGURE 3

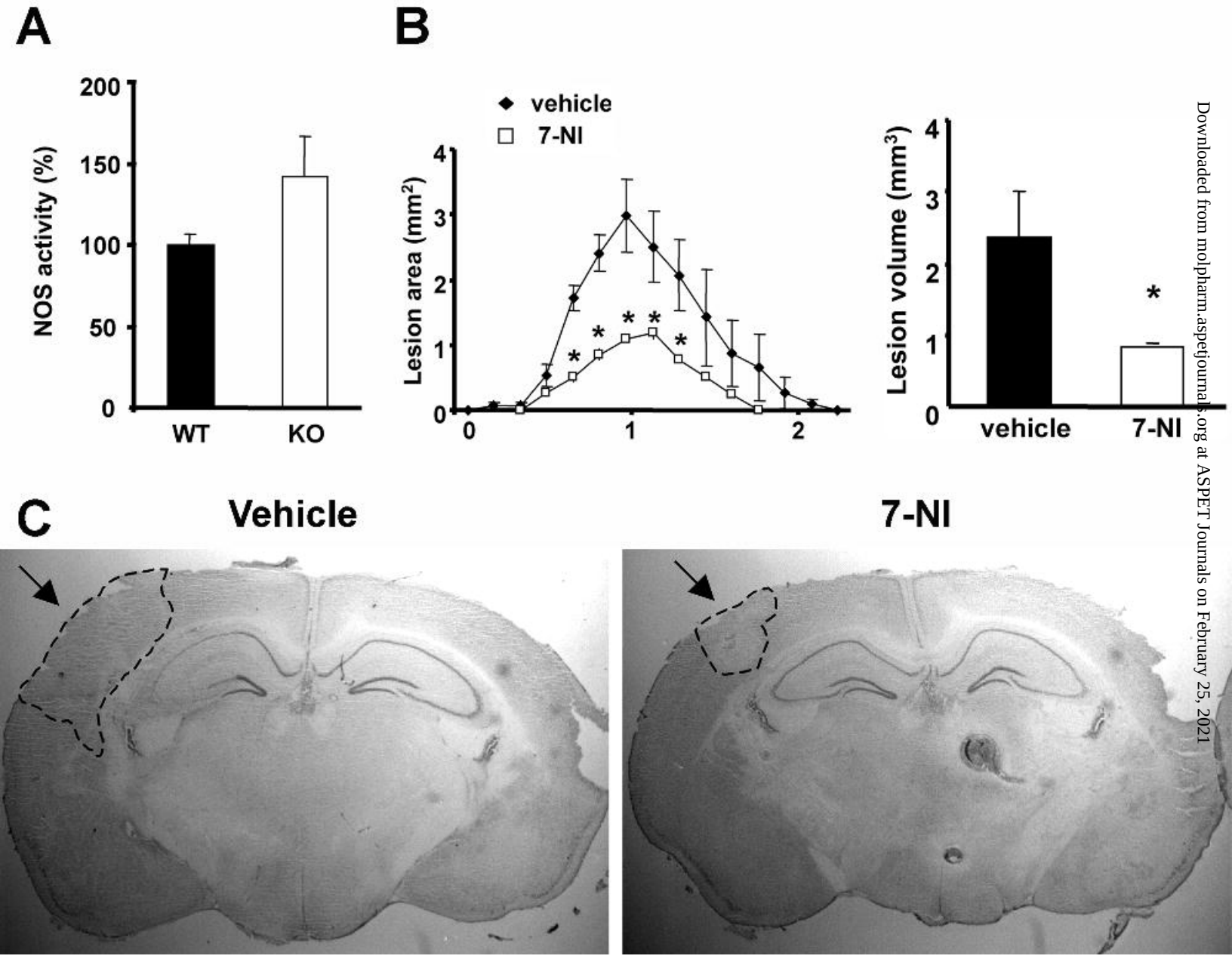

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FIGURE 4

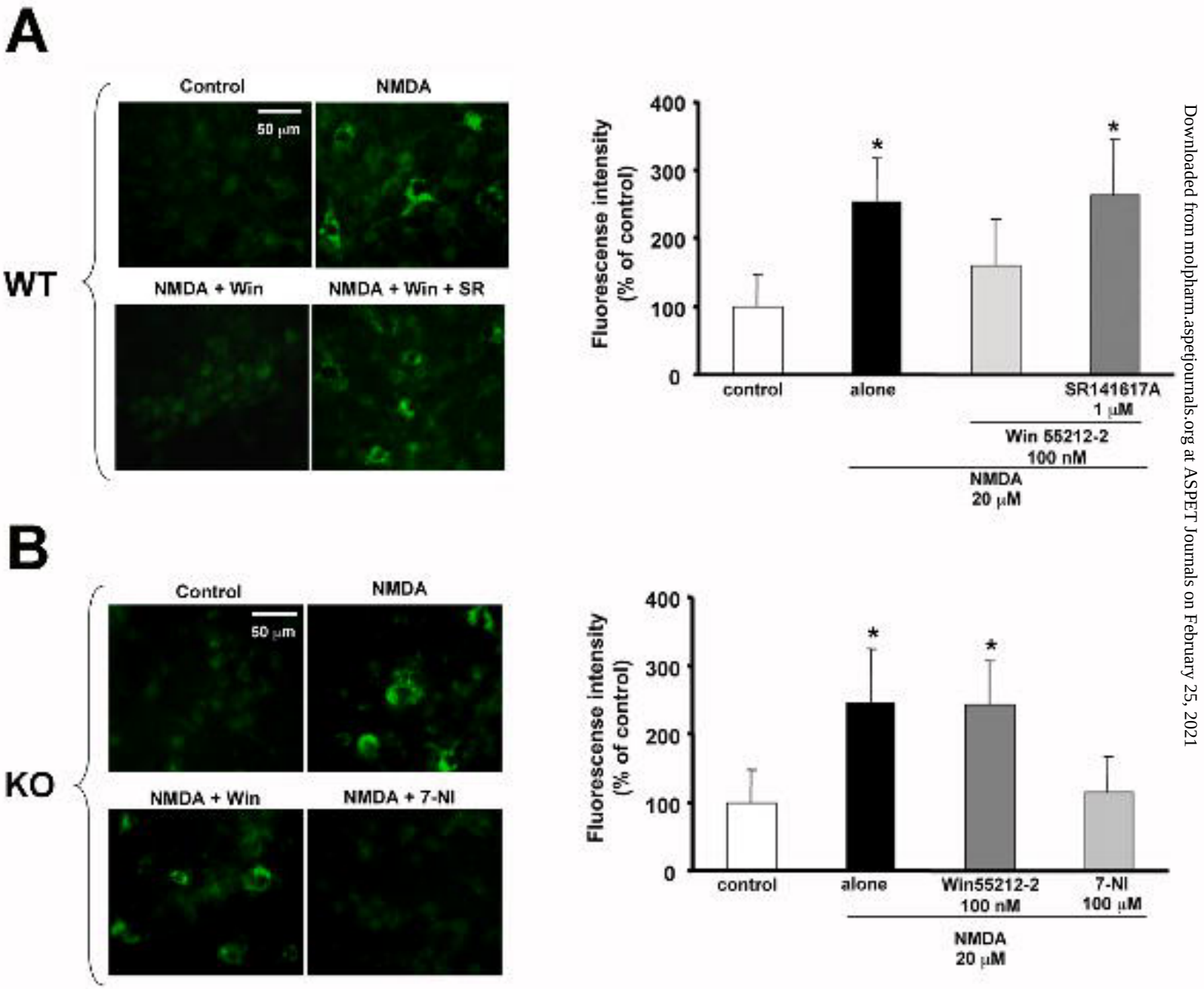

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FIGURE 5