ORIGINAL RESEARCH COMMUNICATION

Cannabinoid Receptors Couple to NMDA Receptors to Reduce the Production of NO and the Mobilization of Zinc Induced by Glutamate

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Abstract

Aims: Overactivation of glutamate *N*-methyl-D-aspartate receptor (NMDAR) increases the cytosolic concentrations of calcium and zinc, which significantly contributes to neural death. Since cannabinoids prevent the NMDAR-mediated increase in cytosolic calcium, we investigated whether they also control the rise of potentially toxic free zinc ions, as well as the processes implicated in this phenomenon. **Results:** The cannabinoid receptors type 1 (CNR1) and NMDARs are cross-regulated in different regions of the nervous system. Cannabinoids abrogated the stimulation of the nitric oxide-zinc pathway by NMDAR, an effect that required the histidine triad nucleotide-binding protein 1 (HINT1). Conversely, NMDAR antagonism reduced the analgesia promoted by the CNR1 agonist WIN55,212-2 and impaired its capacity to internalize CNR1s. At the cell surface, CNR1s co-immunoprecipitated with the NR1 subunits of NMDARs, an association that diminished after the administration of NMDA *in vivo* or as a consequence of neuropathic overactivation of NMDARs, both situations in which cannabinoids do not control NMDAR activity. Under these circumstances, inhibition of protein kinase A (PKA) restored the association between CNR1s and NR1 subunits, and cannabinoids regained control over NMDAR activity. Notably, CNR1 and NR1 associated poorly in HINT1^{-/-} mice, in which there was little cross-regulation between these receptors. **Innovation:** The CNR1 can regulate NMDAR function when the receptor is coupled to HINT1. Thus, internalization of CNR1s drives the co-internalization of the NR1 subunits, neutralizing the overactivation of NMDARs. **Conclusion:** Cannabinoids require the HINT1 protein to counteract the toxic effects of NMDAR-mediated NO production and zinc release. This study situates the HINT1 protein at the forefront of cannabinoid protection against NMDAR-mediated brain damage. *Antioxid. Redox Signal.* 19, 1766–1782.

Introduction

THE NMDA-TYPE ionotropic glutamate receptor permeates calcium into the postsynapse and regulates essential processes in the central nervous system (CNS), such as synaptic plasticity, learning, memory formation, and cognition. Multiple diseases, including stroke, head trauma, epilepsy, dementia, schizophrenia, major depression, Parkinson's and Huntington's diseases, and neuropathic pain, are accompanied by disturbances of *N*-methyl-D-aspartate receptor (NMDAR) signaling (39, 43, 49, 54). Mechanistically, dysregulation of NMDARs, and in particular their overactivation,

results in excessive rises of cytoplasmic calcium, which were initially thought to underlie the fundamental processes that ultimately provoke excitotoxic neuronal death (52). However, increases in nitric oxide (NO) levels, the production of free-radicals/reactive oxygen species (ROS), and oxidative/nitrosative release of endogenous zinc ions are also determinants of NMDAR-dependent excitotoxicity and the ensuing programmed cell death (3, 33, 63).

Several studies have shown how the cannabinoid receptors type 1 (CNR1) counteracts the activity of NMDARs (40), enabling cannabinoids to offer protection from excess NMDAR activity (31, 32, 40). This functional connection would also

Innovation

N-methyl-D-aspartate receptor (NMDAR) dysfunction often accompanies nervous system disturbances (e.g., neurodegeneration, neuropathies, or mental illnesses). We show how cannabinoids prevent NMDAR from driving the nitric oxide overproduction and zinc release that contributes to neurotoxicity. When CNR1s couple to NMDAR NR1 subunits *via* histidine triad nucleotide-binding protein 1 (HINT1) proteins, cannabinoids stimulate their co-internalization, providing neuroprotection by reducing the number of functional NMDARs. Cannabinoid regulation of NMDAR function is lost in the absence of HINT1 or when protein kinase A (PKA) activity disconnects NMDARs from CNR1s. These findings account for the preventive effects of the cannabinoids that act through CNR1, highlighting the need to devise therapeutic strategies which preserve or restore CNR1-NR1 coupling to improve the efficacy of cannabinoids in counteracting disease-associated imbalances in NMDAR.

influence the perception of noxious stimuli during glutamatergic activation, and, consequently, experimental impairment of CNR1 function provokes NMDAR-dependent allodynia and hyperalgesia (56). More relevantly, in neuropathies where NMDAR hyperactivity diminishes the antinociceptive capacity of strong analgesics such as opioids, cannabinoids may still display some of their analgesic effects (5). Since cannabis can promote psychosis and circumstantially precipitate symptoms of schizophrenia, it is considered a drug with a negative cognitive influence when smoked by abusers (12, 15), although such effects are habitually restricted to subjects exhibiting an associated vulnerability (8, 26). Unfortunately, these potentially negative effects have hampered progress in the therapeutic use of cannabinoids to control NMDAR signaling and to mitigate neuronal damage, as well as allodynia and/or hyperalgesia.

CNR1 is the cannabinoid receptor that is mainly implicated in NMDAR regulation (13, 40, 47, 65), and its activation can produce long-lasting neurochemical and functional changes in this glutamatergic system. In rats, prenatal exposure to CNR1 agonists causes a series of alterations in cortical NMDAR signaling in the offspring that affect their cognitive function (4). Moreover, repeated exposure to Δ^9 -THC impairs hippocampal LTP of excitatory glutamatergic transmission, and it diminishes the expression of NMDARs (14). While CNR1s are abundant at presynaptic sites, they are also present at postsynapses of both spinal (28, 53, 60) and supraspinal structures (34, 57). In this context, CNR1s and NMDARs co-localize on neuronal bodies and dendritic processes in certain areas of the nervous system (44), a co-localization in the same cellular compartment that is suggestive of a functional interaction. Several studies have analyzed whether CNR1 activation protects from NMDAR-mediated neurotoxicity, stimulating the removal of excess cytosolic calcium. However, cannabinoids prevent the endogenous increase in calcium through mechanisms related to the direct inhibition of NMDAR calcium influx (40, 69), as also suggested using whole-cell patch clamp recording techniques (38). Thus, besides interacting with distant signaling pathways, cannabi-

noids can also directly affect the open probability of the NMDAR calcium channel.

The processes that connect cannabinoid CNR1 activation with the inhibition of NMDAR excitotoxicity remain ill defined in spatiotemporal terms. The histidine triad nucleotide-binding protein 1 (HINT1) is essential for Mu-opioid receptor (MOR)-NMDAR cross-regulation (58), and this HINT1 protein also associates with the neural CNR1 (20, 61). Hence, we investigated the relevance of HINT1 proteins in the capacity of CNR1s to counteract toxic increases of cytosolic free zinc ions produced by NMDAR over-activation. Our results indicate that cannabinoids selectively neutralize NMDAR-mediated overproduction of NO and the subsequent release of zinc ions, acting on CNR1-HINT1-NMDAR complexes that are sensitive to protein kinase A (PKA)-mediated disruption.

Results

Cannabinoids inhibit the release of zinc mediated by NMDAR through an interaction with HINT1

Signaling through the CNR1 or NMDAR increases the cytosolic levels of free ions, such as calcium or zinc (40, 52, 61, 63). However, the release of endogenous calcium ions persists even when cannabinoids interact with activated NMDARs (25, 40), questioning the relevance of cytosolic calcium clearance in the neuroprotective effect that this interaction provokes (63). Thus, we first explored whether cannabinoids prevent NMDARs from activating the neural nitric oxide synthase (nNOS)/NO cascade that is responsible for the oxidative release of endogenous zinc ions.

Incubation of mouse brain slices with NMDA or different G-protein-coupled receptor (GPCR) agonists, cannabinoids included, increased Newport Green fluorescence (Figs. 1 and 2), a measure of the zinc ion release from endogenous stores (61). As expected, NMDAR-mediated zinc release was attenuated by MK801, and it was also prevented by NOS inhibition. Cannabinoid receptor agonists and NMDA alone mobilize zinc ions; however, their co-administration produced no such effect, although methanandamide was weaker than WIN55,212-2 or CP55,940 in this paradigm. In their interaction with NMDA, agonists of MORs, dopamine D1, D2 receptors, α 2A receptors or serotonin 5HT1A receptors did not impair the NO-mediated zinc release (Fig. 2). These observations indicate that cannabinoids are unique, as their interaction with activated NMDARs blocks the NMDAR and CNR1-stimulated NO production and zinc mobilization.

The interaction between CNR1s and NMDARs appears to be spatially restricted, as the stabilization of NMDARs by antagonists such as MK801 brings about reductions in the analgesic potency of cannabinoids (23, 65). Moreover, in the Newport Green paradigm, NMDAR antagonism also reduced the capacity of WIN55,212-2 to promote zinc release (Fig. 1). The absence of HINT1 did not significantly alter the capacity of GPCR agonists to release zinc ions. However, in such circumstances, cannabinoids were prevented from disrupting NMDA-evoked zinc release (Figs. 1 and 2).

Interactions between CNR1, HINT1, and NR1

Given that CNR1s and NMDARs may co-localize at postsynapses in the brain, we assessed whether they associate in mouse brain synaptosomes. CNR1s co-immunoprecipitated

with NR1 subunits but not, or only minimally, with NR2/3 subunits. Similarly, the NR1 subunit co-immunoprecipitated with CNR1s, and this pattern was evident in various areas of the mouse CNS (although only data for the frontal cortex are shown, Fig. 3A). We addressed the possible direct physical interaction between CNR1s and NR1 subunits. Since the GPCRs reported to form complexes with NR1 subunits establish these interactions through their respective C-termini (16, 55), we first studied the role of CNR1 C-terminal region in its interaction with NR1 subunits.

The C terminus of the NR1 subunit includes the C0-C1-C2(C2') regions (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars), although some NR1 variants lack the regulatory C1 segment (71). In a normal cellular environment (64), direct protein interactions were detected in Chinese hamster ovary (CHO) cells transfected with plasmids expressing CNR1 and NR1 (C0-C1-C2: a 1:1 mix) by bimolecular fluorescence complementation (BiFC). The fluorescent signal displayed by numerous cells indicated that CNR1 and NR1 formed heteromers *in vivo* (Fig. 3B). Using surface plasmon resonance (SPR) analysis to detect protein interactions, we observed an association between the recombinant C1 region of the NR1 C terminus and the CNR1 C-terminal sequence. Indeed, the

CNR1 C terminus bound to the NR1 cytosolic sequence C0-C1-C2 but not to the NR1 C0-C2 *in vitro* (Fig. 3B).

The HINT1 protein exists as a homodimer in cells, and it was initially shown to associate with the C-terminal region of the MOR (24, 58). However, HINT1 may also associate with the CNR1 (20, 61), and when we used BiFC to assess this possibility, a direct physical interaction between HINT1 and CNR1 was evident in living cells. Moreover, SPR analysis and *in vitro* pull-down studies indicated that this interaction was supported by the C-terminal region of the CNR1 (Fig. 4A). The possible binding between HINT1 and NR1 subunit was also evaluated in a similar manner, again indicating that these proteins may associate directly, an interaction requiring the C1 segment of the NR1 subunit (Fig. 4B).

Cross-regulation between CNR1s and NMDARs at the molecular level

A single intracerebroventricular (icv) injection of WIN55,212-2 promoted the internalization of CNR1s (20), although most of them recycled to restore their levels at the cell surface (Fig. 5A). At the cell membrane, CNR1s co-immunoprecipitated with PSD95 proteins, which is further evidence of their presence at the postsynapse, as well as with NR1 subunits and HINT1 proteins, indicative of their association with the NMDAR complex. Internalized CNR1s associated with the HINT1 protein, but they were no longer in

FIG. 1. Effect of GPCR agonists on zinc mobilization from endogenous stores. Coronal mouse frontal cortex slices from WT and HINT1^{-/-} mice were oxygenated and preloaded for 1 h with 50 μM of the cell-permeable Newport Green diacetate, assaying the preparations after its removal. The evolution of spontaneous endogenous zinc release was determined in Control (no treatment) sections, obtaining fluorescent images at various intervals. Images were obtained by confocal microscopy through a 10×0.4 HC PL APO objective on a Leica DMIII 6000 CS confocal fluorescence microscope equipped with a TCS SP5 scanning laser (excitation, 488 nm; emission, 498–520 nm). The cortical regions studied are indicated as A, B, or C, and the data shown were obtained 30 min post-treatment (for each treatment, the images on the left are the controls obtained before adding the compounds under study). The agonists used were: cannabinoids at CNR1s WIN55,212-2, CP55,940, and methanandamide; morphine (MOR); quinpirole (D2R); apomorphine (D1R and D2R); clonidine (α2A); and DPAT (5HT1A). The CNR2 antagonist JTE907 (up to 10 μM; Tocris, 2479) did not alter the capacity of cannabinoids to promote zinc release (not shown); thus, their effects were mostly mediated through CNR1. The NMDAR antagonist MK801 was co-administered with WIN55,212-2. For each agonist, the assays on WT and HINT1^{-/-} mice were performed during the same run. The assays were done in triplicate, and the data were analyzed to determine the means of their luminosity (grayscale of 256 channels; 0 black and 255 white) and 95% confidence intervals (Sigmaplot/Sigmastat v12). For details, see Materials and Methods. Representative images were color indexed and presented in pseudocolor. Scale bar = 500 μm. CNR1, cannabinoid receptors type 1; DPAT, (±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide; GPCR, G protein coupled receptor; HINT1, histidine triad nucleotide-binding protein 1; NMDAR, N-methyl-D-aspartate receptor; WT, wild type. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 2. Cannabinoids produce HINT1-dependent hypofunction of NMDARs: their effect on NMDA-mediated nNOS/NO activation and zinc release. NMDAR-mediated production of NO and the subsequent mobilization of zinc ions. The data shown were obtained at 30 min post-treatment. Control: baseline vehicle; (A): agonist 3 μ M NMDA; MK801 + A: 3 μ M MK801 + 3 μ M NMDA; L-NNA + A: 10 μ M L-NNA + 3 μ M NMDA. Influence of cannabinoids and of agonists at other GPCRs on the release of zinc promoted by NMDA. Different sections were used to analyze the effect of NMDA when administered alone or in combination with the GPCR agonists. The CNR1 cannabinoid antagonist Ly320135 (3 μ M) was used to diminish the effects of WIN55,212-2 (see Fig. 1 for details). L-NNA, NG-Nitro-L-arginine; nNOS, neural nitric oxide synthase; NO, nitric oxide. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

contact with the NR1 subunits. Notwithstanding, WIN55,212-2 also stimulated the loss of surface NR1 subunits that were internalized and then separated from the CNR1s (Fig. 5B).

While the NMDAR antagonist, MK801, did not affect the association of surface CNR1s with NR1 subunits, HINT1 proteins, or PSD95 proteins, this antagonist reduced WIN55,212-2-stimulated internalization of CNR1s and NR1 subunits (Fig. 5C, D). These observations suggested that the CNR1-NR1 association was altered by the binding of antagonists to the NMDAR. In fact, unlike the competitive antagonist D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5), noncompetitive NMDAR antagonists, MK801 or ifenprodil, added NR2 subunits to the CNR1-NR1 complex (data for MK801 are shown in Fig. 6A). Thus, MK801 and Ifenprodil promoted the coupling of the entire NMDAR to the CNR1 *via* NR1 subunits. This increase in cargo impaired CNR1 internalization and, as a consequence, their resensitization-recycling,

which probably influenced the capacity of cannabinoids to produce analgesia.

The possible role of HINT1 in the interaction between CNR1 and NMDAR was determined using a protocol of repeated activation of the CNR1. Three identical and consecutive doses of WIN55,212-2 administered 90 min apart considerably reduced the presence of CNR1s and NR1 subunits at the cell membrane, while augmenting their co-internalization (Supplementary Fig. S2; Fig. 6B). As observed for a single dose of WIN55,212-2, MK801 also diminished the loss of surface CNR1s and NR1 subunits in this demanding protocol. In the absence of the HINT1 protein, the influence of NMDARs on CNR1 activity disappeared, and MK801 did not prevent the loss of surface CNR1 subunits induced by repeated WIN55,212-2 administration. Notably, in HINT1^{-/-} mice, WIN55,212-2 barely induced the co-internalization of NR1 subunits (Fig. 6C).

FIG. 3. CNR1 and NMDAR associate in the nervous system and interact physically *in vitro*. (A) Solubilized cortical synaptosomes were incubated with biotinylated IgGs directed against the first extracellular loop of CNR1. After recovery with streptavidin-sepharose, the CNR1-containing complexes were processed to remove the IgGs before detecting any associated proteins (see Materials and Methods). The antibodies used to detect NR subunits were NR1, ab1880; NR2A, ab14596; NR2B, ab14400; NR3AB, ab2639. The antibody directed against the NR1 extracellular peptide sequence (Sigma-Genosys; 482–496) was used to co-immunoprecipitate CNR1. IP stands for immunoprecipitation. (B) BiFC analysis of the interaction between the CNR1 and NR1 subunits. CHO cells were transiently co-transfected with cDNAs encoding NR1VN173 and CNR1VC155 (0.3 μ g), and confocal fluorescent signals were obtained when NR1VN173 and CNR1VC155 associated. For each pair of images: *Left*, Phase field and fluorescent images are combined; *Right*, Fluorescent image of cells in the field. SPR analysis of the CNR1-NR1 interaction. The CNR1 C-terminal sequence (401–473) interacts with the NR1 C-terminus containing the C1 region. Sensorgrams were constructed with CNR1 Ct in fluid phase at the concentrations indicated (μ g/ml), and no signal was detected with NR1 C0–C2. NR1 C0–C1–C2 or NR1 C0–C2 C-terminus variants were incubated with the GST-CNR1 C-terminus (401–473) or GST alone (control), and (P) the GST complex was captured with glutathione sepharose and probed in western blots (WB) with an antibody against the NR1 C2 region (ab6485). The CNR1 C-terminal sequence co-immunoprecipitated with NR1 C0–C1–C2 but not with NR1 C0–C2, and GST did not bind to the NR1 C1 segment. BiFC, bimolecular fluorescence complementation; SPR, surface plasmon resonance.

Functional relevance of the CNR1-HINT1-NMDAR association

Given the possibility that CNR1s control the activity of NMDARs *via* HINT1, we analyzed what influence such an association may have in several physiological and pharmacological situations in which CNR1s participate. Cannabinoids injected into supraspinal structures reduce nociceptive transmission at the spinal level through descending pathways (23). Indeed, WIN55,212-2 produced dose-dependent analgesic effects when injected icv into wild-type (WT) mice and those lacking the HINT1 protein. More significantly, the antagonism of NMDARs revealed profound differences in the regulation of cannabinoid analgesia by WIN55,212-2 in both groups of mice. In WT mice, noncompetitive NMDAR

antagonists (MK801 and Ifenprodil) greatly reduced WIN55,212-2 and methanandamide analgesia, while a competitive antagonist (D-AP5) produced a weaker effect that was not statistically significant. However, NMDAR antagonists did not affect the analgesia produced by cannabinoids in HINT1^{-/-} mice, probably due to the disruption of the association between CNR1 and NMDAR (Fig. 7A and Supplementary Fig. S3). These data indicate that the cannabinoids produce analgesia independently of the CNR1-NMDAR connection, although this relationship enables NMDAR antagonists to impair CNR1 recycling and cannabinoid analgesia.

The absence of the HINT1 protein provoked an increase in the capacity of NMDA to promote the death of cultured cortical neurons. While WIN55,212-2 protected HINT1^{+/+}

A CNR1 Ct – HINT1 interaction**B** HINT1-NR1 C1 interaction

FIG. 4. The HINT1 protein binds to the CNR1 C-terminal sequence and the C1 segment of the NMDAR NR1 subunit. **(A)** The HINT1 protein interacted with the CNR1 C-terminal sequence, as evident by SPR and co-precipitation *in vitro*. **(B)** Direct physical association between HINT1 and NMDAR NR1 subunit. For BiFC analysis of the interaction between CNR1/NR1 and HINT1, CHO cells were transiently co-transfected with cDNAs encoding CNR1VC155/HINT1VN173 and NR1VN173/HINT1VC155 (0.3 μ g). Details as in Figure 3.

neurons against such an NMDA insult, this treatment did not protect HINT1^{-/-} neurons (Fig. 7B). In the absence of HINT1, cultured neurons showed increased cytosolic calcium levels in response to NMDA, and while cannabinoids reduced the NMDAR-mediated influx of calcium in HINT1^{+/+} cells, such control was absent in HINT1^{-/-} cultured neurons (Fig. 7C, Supplementary Fig. S4). Molecular studies indicate that HINT1 proteins determine the quality of the CNR1-NR1 interaction, as CNR1 co-immunoprecipitated less NR1 in the absence of HINT1 even though WT and HINT1^{-/-} mice expressed similar amounts of CNR1 and NR1 subunits (Fig. 7D). Thus, cannabinoid agonists negatively control NMDAR activity when CNR1s are coupled *via* HINT1 proteins to the NR1 subunits (Fig. 7).

CNR1 signaling is regulated by a series of serine and threonine kinases (37), and, thus, we evaluated how selective kinase inhibitors affect WIN55,212-2 analgesia, as well as

explored their influence on the association between CNR1 and NR1 subunits. PKC inhibition (1 nmol Gö7874 or Chelerythrine) enhanced WIN55,212-2 antinociception, as did KN93 (7 nmol) or KN62 (10 nmol), CaMKII inhibitors. However, no significant effect on WIN55,212-2 analgesia was produced by the PKA inhibitor, 6-22 amide (15 nmol, data not shown). Accordingly, we addressed the influence of PKC and CaMKII inhibitors on the reduction of WIN55,212-2 analgesia produced by NMDAR antagonism. Both PKC and CaMKII inhibitors prevented the antagonism of WIN55,212-2-evoked analgesia produced by MK801 (Fig. 8A and Supplementary Fig. S5).

At the molecular level, and in the absence of pharmacological interventions affecting CNR1s or NMDARs, the *in vivo* inhibition of PKC or CaMKII did not alter the *ex vivo* co-immunoprecipitation of CNR1s with NR1 subunits. By contrast, the PKA inhibitor significantly enhanced this

FIG. 5. The antagonism of NMDARs impairs the capacity of Win55,212-2 to internalize CNR1s. **(A)** Mice received an icv dose of WIN55,212-2 (20 nmol). Groups of mice were then sacrificed at the time points indicated, and the synaptosomes obtained from the frontal cortex were processed into membrane and soluble fractions (see Materials and Methods). CNR1 was immunoprecipitated, and the membrane and internalized CNR1 and its co-immunoprecipitated proteins were assessed in western blots. $^*/\phi$ Significantly different from the control group (C membrane/C' internalized) that did not receive the cannabinoid. **(B)** Mice that received icv WIN55,212-2 (20 nmol) were processed as described in **(A)**. The membrane and internalized NR1 subunits were immunodetected directly in western blots. $^*/\phi$ Significantly different from the corresponding membrane or internalized control (0 min) that did not receive the cannabinoid. **(C, D)** Mice received an icv dose of WIN55,212-2 (20 nmol) along with MK801 (1 nmol) and were processed as in **(A)** and **(B)**.

association, revealing a tonic negative control exerted by PKA on the CNR1-NMDAR interaction (Fig. 8B). We also studied the effect of these kinase inhibitors on the interference of WIN55,212-2-induced internalization of CNR1s provoked by MK801. The *ex vivo* analysis revealed that the inhibition of PKC/CaMKII restored the capacity of WIN55,212-2 to internalize CNR1s in mice administered MK801, although there was virtually no increase in the NR1 subunits internalized. Conversely, PKA inhibition more weakly overrode the inhibition of WIN55,212-2-induced CNR1 internalization by MK801, although it promoted a strong increase in the NR1 subunits internalized (Fig. 8B). Thus, PKC and CaMKII activities are recruited in CNR1 activation to enhance the association between CNR1 and NR1 (and that of CNR1-NR1/NR2 in the presence of MK801). As such, the inhibition of PKC prevented WIN55,212-2 from offering protection against NMDA neurotoxicity in cortical cultured neurons (Supplementary Fig. S6). CaMKII is an effector of NMDARs, and its inhibition reduced NMDA-mediated cell death. Thus, the influence of CaMKII inhibition on the CNR1-NMDAR relationship could not be assessed in this cellular model. Con-

versely, the inhibition of these kinases weakened the CNR1-NR1 interaction, facilitating analgesia by cannabinoids even in the presence of NMDAR antagonism (as observed in the absence of HINT1). PKA mostly acted on NR1 subunits, thereby separating NMDARs from CNR1s. Accordingly, PKA inhibition did not increase analgesia but, rather, it augmented the association between these two receptors. In such circumstances, and in the presence of MK801, CNR1 internalization remained impaired but those CNR1 that internalized carried more NR1 subunits. Our results highlight the different roles of serine and threonine kinases in the regulation of NMDAR activity by CNR1, which is consistent with previous observations defining a different role for PKC/CaMKII to PKA in the potentiation of excitatory postsynaptic NMDAR-mediated currents (36).

One attractive possibility is that HINT1 *via* CNR1 exerts a negative influence on NMDAR function, a hypothesis supported by the enhanced excitotoxic effects of NMDA in HINT1^{-/-} cortical neurons (Fig. 7B). Thus, the stability of the CNR1-NMDAR association was analyzed in response to NMDA and neuropathic pain, a situation in which NMDARs

FIG. 6. HINT1 protein determines the control of the NMDAR by CNR1 internalization. (A) MK801 stabilizes the association between NR subunits at the NMDAR. The NMDAR antagonist MK801 (1 nmol, icv) was administered 30 min before sacrificing the mice. The CNR1 was immunoprecipitated from brain synaptosomes, and the co-immunoprecipitation of NR1 and NR2 subunits was then assessed in western blots. (B, C) WT and HINT1^{-/-} mice received three consecutive icv doses of WIN55,212-2 (20 nmol) spaced 90 min apart, alone or along with MK801 (1 nmol). The mice were then sacrificed 3 h after the last injection, CNR1 was immunoprecipitated from the membrane and soluble fraction of cortical synaptosomes, and the content of CNR1 and NR1 subunits was determined. *Significantly different from the corresponding control value that did not receive the cannabinoid.

are overactivated (42). The activity of the NMDAR/CaMKII pathway was enhanced in mice suffering from chronic constriction injury for 7 days (Supplementary Fig. S7), and the association of CNR1s to NR1 subunits diminished. A similar situation was observed 6 h after the icv administration of NMDA (Fig. 9A), linking these phenomena directly to NMDAR activation (see scheme in Fig. 9). PKA activation disrupts the CNR1-NMDAR connection and prevents cannabinoids from protecting against NMDA neurotoxicity (32). Accordingly, the inhibition of PKA or NMDAR antagonism that alleviates the negative effects of the neuropathic pain syndrome (6, 51) also restored CNR1-NMDAR association, diminishing the NMDAR-related CaMKII activity.

Cannabinoids prevent or protect against excitotoxicity when they are given before or after but close to NMDAR

activation. As the interval between NMDAR activation and cannabinoid administration increases, neuroprotection diminishes (69). Considering that the activation of NMDARs separated CNR1s from these receptors, we studied whether the order of WIN55,212-2 and NMDA administration influenced the co-internalization of NR1 subunits. Thus, WIN55,212-2 administered before NMDA promoted a more intense loss of surface NR1 subunits than that observed when NMDA preceded WIN55,212-2 (Fig. 9B). Moreover, the latter protocol augmented the internalization of CNR1s. PKA activation inhibits the protection that CNR1 agonists offer against NMDA toxicity (32); it is possible that this might be, in part, mediated by disrupting the CNR1-NR1 association, as PKA inhibition was seen to enhance this interaction.

FIG. 7. Antagonism of WIN55,212-2-induced supraspinal analgesia by MK801: the influence of the HINT1 protein. **(A)** The effects of various icv doses of WIN55,212-2 were studied in mice lacking the HINT1 protein and in their WT littermates. Each point is the mean $\pm$ SEM from groups of at least six mice. Both WT and HINT1-deficient mice showed comparable responses to increasing doses of WIN55,212-2. The NMDAR antagonist, MK801 (1 nmol), was icv-injected to the mice 30 min before WIN55,212-2. *Significantly different from the group that received saline instead of MK801; $p < 0.05$. **(B) Left panel,** Cortical cell cultures from wild-type and HINT1^{-/-} mice were exposed to increasing concentrations of NMDA for 24 h. Cell death was measured by LDH efflux into the medium. The data shown are the mean $\pm$ SEM from 20 wells per group. *Significant difference between wild-type and HINT1^{-/-} cultured neurons, $p < 0.05$. **Right panel,** cultures were exposed to a fixed concentration of 50 μ M NMDA for 24 h in the presence or absence (–) of increasing concentrations of the cannabinoid agonist WIN55,212-2. To rule out the possible participation of CNR2, the assay was conducted in the presence of 3 μ M of the CNR2 antagonist JTE907. *Significant difference with regard to NMDA alone, $p < 0.05$. (1–4) Fluorescence photomicrographs of cortical cell cultures immunolabeled with an anti-MAP2ab. (1) WT: 50 μ M NMDA; (2) WT: 50 μ M NMDA plus 100 nM WIN55,212-2; (3) HINT1^{-/-}: 50 μ M NMDA; and (4) HINT1^{-/-}: 50 μ M NMDA plus 100 nM WIN55,212-2. **(C)** NMDA-induced changes in [Ca²⁺]_i concentration within the cells. Bar graph indicates the mean $\pm$ S.E.M fluorescence ratio (494/523 nm) averaged across 10–15 cells per condition. HINT1^{+/+} and HINT1^{-/-} cultures were exposed during 2 min perfusion to NMDA (15 μ M) alone or in combination with MK801 (10 μ M) and WIN55,212-2 (100 nM). Cells were incubated in artificial cerebrospinal fluid (ACSF). *Significant difference relative to NMDA, ϕ between wild-type and HINT1^{-/-} cultured neurons, $p < 0.01$. **(D)** The absence of HINT1 reduces the *in vivo* association between the CNR1 and NR1 subunits. The CNR1 was immunoprecipitated from solubilized cortical synaptosomes or cortical cell cultures obtained from WT and HINT1^{-/-} mice, and the CNR1 and co-immunoprecipitated NR1 was assessed. The NR1 content is shown.

FIG. 8. PKC, PKA, and CaMKII regulate WIN55,212-2 analgesia and the CNR1-NR1 association. (A) Effect of PKC and CaMKII inhibition on WIN55,212-2 analgesia. Mice received the PKC inhibitor Gö7874 (1 nmol) or the CaMKII inhibitor KN93 (7 nmol) icv, 30 min before the cannabinoid agonist (6 nmol). In one set of assays, MK801 (1 nmol) was given 10 min after the kinase inhibitors; then, WIN55,212-2 was injected icv 20 min after the NMDAR antagonist, and the time course of analgesia was determined. *Significantly different from the group that received WIN55,212-2 and saline instead of the kinase inhibitors (*left panel*); or from the groups not receiving MK801 (*right panel*), $p < 0.05$. The efficacy of the doses and intervals used were previously determined for NMDA, MK801, and kinase inhibitors (59). **(B)** Association of CNR1 with the NMDAR. Kinase inhibitors were icv injected into the animals 30 min before sacrifice (1 nmol Gö7874; 5 nmol PKA inhibitor; and 7 nmol KN93); CNR1 was immunoprecipitated from solubilized synaptosomal cortical membranes, and its association with NR1 subunits was determined in western blots. *Significantly different from the control group (C) that received saline instead of the kinase inhibitors, $p < 0.05$. The effect of inhibiting PKC, PKA, or CaMKII on MK801; the impairment of WIN55,212-2-induced co-internalization of CNR1 and NR1 subunits. Kinase inhibitors were icv-injected 30 min after MK801 (1 nmol) and 30 min before WIN55,212-2. The mice were sacrificed 30 min later, and the presence of internalized CNR1 and NR1 subunits was determined. *Significantly different from the control group (C) that received WIN55,212-2 and MK801 but saline instead of the kinase inhibitors, $p < 0.05$. CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; PKA, protein kinase A; PKC, protein kinase C.

Discussion

Cannabinoids are widely recognized for their proven efficacy in enhancing cell viability in different models of neurotoxicity. Since their positive effects are not always related to CNR1 or CNR2 activation (27, 48), our study should be considered in the framework of the mechanisms used by cannabinoids to control NMDAR hyperactivity. The overactivation of postsynaptic NMDARs is apparently behind a variety of neuropathologies (39, 54), and cannabinoids probably diminish the impact of NMDAR excitotoxicity through different mechanisms, such as the activation of presynaptic CNR1s to reduce glutamate release into the cleft (7, 38, 50), or that of postsynaptic cannabinoid receptors whose signaling pathways could interfere with those of NMDARs (25, 40). However, there are observations linking CNR1 activation with the direct inhibition of NMDAR calcium influxes (40).

Our study indicates that cannabinoids inhibit NMDAR activity through the association of CNR1s with NMDAR NR1 subunits *via* HINT1 proteins. In this scenario, cannabinoid agonists disassemble NMDARs through the co-internalization of CNR1s with NR1 subunits, thereby reducing neurotoxicity. The HINT1 protein is widely expressed in nervous tissue (41), and it is dysfunctional in a series of neural illnesses. The HINT1 gene is located on chromosome 5q31.2, a region in which linkage studies have implicated in schizophrenia, as well as being associated with psychosis (67, 68); its mRNA expression is altered in postmortem brains from schizophrenia patients (11). Moreover, mutated forms of the HINT1 protein have been identified in association with neurodegenerative disorders, such as axonal neuropathy with neuromyotonia (70). Since HINT1 can associate with several GPCRs (61), this protein probably influences their function, as well as the homeostatic connection of CNR1s with NMDARs.

FIG. 9. Plasticity of the CNR1-NR1 association. (A) Influence of chronic constriction injury (CCI) and direct NMDA administration to mice on NR1 co-immunoprecipitation with CNR1s. Mice suffering from CCI were sacrificed on day 7, and the CNR1-NR1 association was determined in membranes isolated from cerebral cortex synaptosomes. A group of mice also received 5 nmol of the PKA inhibitor 6-22 amide 30 min before they were sacrificed. A similar study was performed after icv injecting NMDA (15 pmol per mouse) and assessing the CNR1-NR1 association in synaptosomes 24 h later. The presence of CaMKII and of its P-Thr286 form is shown. For each assay (column), the cortical structures from six mice were pooled for the *ex vivo* determinations. C and IP stand for control mice and immunoprecipitation, respectively. *Significantly different from the Sham-operated control group or mice not receiving NMDA (C), $p < 0.05$. (B) The sequence of WIN55,212-2 and NMDA icv injection influences the internalization of CNR1 and NR1 subunits. The mice received 20 nmol WIN55,212-2 and 15 pmol NMDA using the protocols indicated. The mice were sacrificed 90 min after NMDA or 30 after WIN55,212-2, and the levels of CNR1 and NR1 subunits were determined. *Significantly different from the indicated control group, $p < 0.05$. PKA model: NMDAR activates PKA: Calcium fluxes recruit Calmodulin (CaM) to form Ca^{2+} -CaM, which is necessary to regulate CaMKII (1) and activate adenylyl cyclase types I and VIII. (2) Increased cAMP production activates PKA, which, in turn, inhibits protein phosphatase 1 (PP1: 3) that opposes CaMKII activity. (4) CaMKII consolidates its autophosphorylation on Thr286, which is now being activated independently of Ca^{2+} -CaM levels and ready to regulate CNR1 activity or to remove RGSZ2.G α i/oGTP from HINT1 proteins. Moreover, when PKA acts on certain serines in the NR1 C1, the regulatory segment promotes the separation of CNR1s from NMDARs. Inset: Diagram of the mechanism proposed for WIN55,212-2 induced NMDAR hypofunction. CNR1 and the NMDAR associate in the postsynapse, with PKC and CaMKII enhancing this association, and PKA diminishing it. (1) The agonist binds to CNR1 and (2) promotes co-internalization of CNR1 and NR1 subunits. These proteins separate in the cytosol, and (3) CNR1 returns to the plasma membrane. (4) The re-sensitized CNR1 re-associates with new NR1 subunits, and (5) the cycle starts again, while the agonist continues in the receptor environment. RGSZ2, regulator of G protein signaling 17 (Z2).

The validity of this association is supported by a series of observations, which include the following: the co-immunoprecipitation of CNR1s with NR1 subunits from the postsynapse in mouse brain; the co-internalization of CNR1s with NR1 subunits; the dampened effect of noncompetitive NMDAR antagonists on the capacity of WIN55,212-2 to internalize CNR1s; and also by the fact that cannabinoids which promote strong internalization of CNR1s, for example, WIN55,212-2 and ACEA (20), are better neuroprotectors than those that produce weak internalization (*e.g.*, AEA or anandamide/methanandamide) (32, 35). All these situations were dependent on the HINT1 protein and in its absence, the CNR1-NR1 association weakened, cannabinoids promoted no NR1 internalization, and MK801 failed to alter CNR1 internalization.

PKA activity diminished the CNR1-NR1 association and impaired the capacity of cannabinoids to co-internalize NR1 subunits, whereas PKC and CaMKII increased their connection and favored CNR1/NR1 co-internalization. Moreover, interventions producing the segregation of CNR1s from NMDARs brought about a decrease in the control exerted by cannabinoids on NMDAR-mediated cytosolic increases of calcium and zinc ions, or in their capacity to protect cell viability against NMDA insult (*i.e.*, as produced in the absence of HINT1, by increased PKA activity, or NMDA challenge before cannabinoid arrival).

Besides CNR1, a few GPCRs functionally associate with NMDARs, such as the dopamine D1 receptors (16), metabotropic mGlu5a glutamate receptors (55), and MORs (59). Among these, MORs and CNR1s require HINT1 to maintain their connection with NMDARs operative (58). However, similar assemblies of signaling proteins support different regulatory processes, and while MORs stimulate the activity of the associated NMDARs *via* PKC and Src, CNR1s oppose their function and decrease calcium permeation (10, 21), as well as NO-mediated zinc release. Moreover, morphine promotes the separation of MORs from the NR1 subunits, favoring NMDAR activation; whereas CNR1s co-internalize the associated NR1 subunits to disrupt NMDAR function, and unlike MORs, the internalized CNR1s remain bound to HINT1 (58).

The over stimulation of NMDARs, such as that produced by a direct icv injection of NMDA into mice or that observed in different neuropathies (30, 42), restricts the CNR1-NR1 association, increases NMDAR function, and results in NMDAR-dependent hyperalgesia (56). The activation of NMDARs stimulates the calcium-calmodulin/adenylyl cyclase/cAMP/PKA pathway and promotes the separation of NR1 from HINT1 by phosphorylation of NR1 Ser897 (66). In the absence of HINT1, the neurotoxicity of NMDA increases in cultured cortical cells, as observed in CNR1^{-/-} cells (32). Thus, the binding of the CNR1-HINT1 complex to NR1 subunits appears to play a role similar to that of calcium-calmodulin in the negative control of NMDAR activity (9). In this context, PKA inhibition would reduce allodynia/hyperalgesia (6, 19) by promoting the inhibitory binding of NR1 subunits to HINT1 proteins. In fact, PKA inhibition prevents NMDA antagonism of morphine analgesia and the separation of the MOR/CNR1 from NR1 subunits (59). The existence of this CNR1-NMDAR association could also account for the interference of PKA activity with the efficacy with which cannabinoids act against NMDAR overactivation (32), or on

NMDAR calcium fluxes (25, 40). Cannabinoids diminish NMDAR excitotoxicity when administered shortly before or after activators of NMDAR function, and their efficacy diminished as this interval increased (69). Since PKA impairs the association between CNR1s and NR1 subunits, an increase in the interval between WIN55,212-2 and NMDA administration favored NMDAR-mediated activation of PKA, which now displaces the negative influence of CNR1 activation on NMDAR function.

Cannabinoid analgesia does not require CNR1 and NMDAR coupling; however, when coupled, NMDAR antagonism reduces this antinociception, and this effect depends on HINT1. Thus, NMDAR antagonism greatly reduced cannabinoid-induced analgesia and CNR1 internalization, indicating that cannabinoid effects such as antinociception require the internalization/recycling-resensitization of their CNR1s. Indeed, internalized CNR1s return to the cell surface within a few minutes of agonist challenge (29), and the resensitized CNR1s rapidly associate with the NMDAR NR1 subunits, preparing new NR1 subunits for co-internalization. This mechanism produced the cytosolic accumulation of NMDAR subunits and dampened NMDAR signaling. The negative control that cannabinoids can exert on NMDAR function is certainly relevant. This regulation would operate with endogenous cannabinoids released in response to NMDAR hyper activation (25), although this control apparently fails or is overridden by the activation of the NMDARs in circumstances such as neuropathies and certain neurodegenerative illnesses.

Overactivation of glutamate NMDARs typically produces excess calcium influx and release from internal organelles. It also augments the production of NO, which reacts with superoxide to form peroxynitrite, generating ROS and releasing zinc from intracellular stores on oxidative and nitrosative stimulation. This metal ion contributes significantly to toxicity by damaging critical metabolic enzymes and contributing to the engagement of apoptotic cascades (17, 33). In direct comparison to calcium, zinc homeostasis seems particularly sensitive to oxidative stress, and its de-regulation appears to enhance neurotoxicity, probably because different intensities of zinc load activate distinct harmful pathways (1, 18, 63). Therefore, to protect against NMDAR overactivation, cannabinoids should reduce not only cytosolic calcium levels but also those of zinc. Cannabinoids reduce the primary calcium influx through activated NMDARs and its subsequent release from endogenous stores (25, 63, 69), and we showed that they can efficiently cancel out the NMDA-mediated release of zinc ions. Cannabinoids mainly prevent NMDA-induced rises in cytosolic calcium by acting on the NMDAR rather than by stimulating its clearance by reuptake or its expulsion into the extracellular space (63, 69). Our data indicate that a similar mechanism may also control the levels of zinc.

In fact, there is virtually no release of zinc ions after simultaneous CNR1 and NMDAR activation, making it possible that co-activation of CNR1s and NMDARs cancels out this common effect. However, cannabinoids promote IP3 signaling in their interaction with activated NMDARs, stimulating the release of calcium from intracellular stores and thereby raising cytosolic calcium levels (40). Consequently, to control NMDAR overactivation, some features of CNR1 signaling are lost, such as the production of NO and the release of free zinc ions (61). Since GPCRs, similar to the CNR1, are coupled to

NO production *via* the HINT1-RGSZ2-nNOS complex (2, 22), an attractive possibility is that NR1 subunits and RGSZ2-nNOS complexes compete for HINT1 proteins. Thus, on simultaneous activation of CNR1s and NMDARs, kinases such as PKC or CaMKII disrupt the HINT1/RGSZ2-nNOS association (58), favoring HINT1 binding to NR1 subunits. This phenomenon would couple the formation of CNR1-NMDAR complexes with the level of NMDAR activity, and also uncouple the CNR1 from the source of NO (nNOS) and zinc ions (RGSZ2) (22, 61), preventing cannabinoids from contributing NO/zinc to the process of glutamatergic excitotoxicity. The plasticity of this regulatory process would help adjust the CNR1-NMDAR association to the level of NMDAR activation (45), thereby making the control exerted by cannabinoids over NMDARs more efficient and, importantly, protecting against unnecessary NMDAR hypoactivity (46). This is a possibility worth of further study.

In summary, we describe here the association of CNR1s with NMDAR NR1 subunits at the postsynapse in the mouse brain. *In vitro*, *ex vivo*, and *in vivo* data indicate that this association is stabilized by the HINT1 protein, which enables cannabinoids to prevent the elevated cytosolic levels of calcium, NO, ROS, and free zinc that follow glutamate NMDAR-mediated excitotoxicity, and which compromise cell viability. At the molecular level, the activation of CNR1s promotes their co-internalization with NR1 subunits, producing NMDAR hypo-function. Cannabinoid neuroprotection is overridden in the absence of HINT1 proteins and also by PKA activity, situations that uncouple NMDARs from CNR1. Thus, in order to obtain effective cannabinoid control over NMDAR activity, it is necessary to preserve or restore the CNR1-NR1 association, which can be achieved by inhibiting PKA, stimulating PKC/CaMKII activity, or separating the RGSZ2 bound to HINT1 protein in order to increase the association of the latter with NR1 subunits.

Materials and Methods

Animals, icv injection, and evaluation of antinociception

A mouse knock-out strain with targeted disruption of HINT1 on a 96% 129 mice genetic background (a gift from I.B. Weinstein/J.B. Wang) was used in these studies. The response of the animals to nociceptive stimuli was determined by the warm water (52°C) tail-flick test, and antinociception was expressed as a percentage of the maximum possible effect ($MPE = 100 \times [\text{test latency} - \text{baseline latency}] / [\text{cut-off time} (10 \text{ s}) - \text{baseline latency}]$). The baseline latencies ranged from 2 to 2.5 s, and they were not affected by the kinase inhibitors used or their solvent [see (59, 62)]. Compounds were injected into the lateral ventricle of lightly anaesthetized (ether) mice in a volume of 4 μ l. Saline was used as control, and antinociception was assessed at different time intervals after administration.

All the procedures involving mice were performed in strict accordance with the European Community guidelines for the Care and Use of Laboratory Animals (Council Directive 2010/63/EU).

Zinc-microfluorescence imaging in mouse cortical slices

For intracellular Zn^{2+} imaging, brain slices (200 μ m) containing the frontal cortex (2.50–1.50 mm to bregma) were

preloaded with cell permeant Newport Green DCF diacetate (50 μ M; N7991, Invitrogen), 0.1% pluronic acid, and 0.5% dimethyl sulfoxide for 1 h, as described elsewhere (56, 58). The size and resolution of the images captured (a pixel depth of 8 bits) was identical for each treatment. The grayscale images were then resolved into 256 shades of gray, where 0 is black and 255 corresponds to white. For each image, histograms of the number of pixels with a given shade of gray were plotted and then, the mean luminosity of the control and treated images were computed (AlphaEase FC Software).

Immunoprecipitation and Western blotting

The preparation of membrane and cytosolic fractions, and the immunoprecipitation from brain synaptosomes of CNR1s and NR1 subunits, was performed as previously described (20, 59). The specificity and efficacy of the antibodies used in immunoprecipitation assays has been addressed elsewhere (Supplementary Fig. S1B) (20, 22). The immunocomplexes recovered were resolved by SDS/polyacrylamide gel electrophoresis (PAGE), and the separated proteins were then transferred onto 0.2 μ m polyvinylidene difluoride (PVDF) membranes (BioRad 162-0176), probed with the primary antibodies, and detected using secondary antibodies conjugated to horseradish peroxidase. Antibody binding was visualized by chemiluminescence (GE Healthcare; ECL Prime WBDR, RPN2232) and recorded with a ChemImager IS-5500 (Alpha Innotech). Densitometry was performed using Quantity One Software (BioRad) and expressed as the mean $\pm$ SEM of the integrated volume (average optical density of the pixels within the object area/ mm^2). The data are expressed relative to the levels observed for the control group, attributed an arbitrary value of 1. The assay was repeated typically thrice on samples derived from independent groups of mice, and the results were always comparable. Equal loading was verified and adjusted, if necessary, *versus* actin or the immunoprecipitated receptor.

Expression of recombinant proteins and the evaluation of CNR1-NMDAR1 interactions

The C-terminus of CNR1 was amplified by RT-PCR using total RNA isolated from mouse brains. The PCR products were cloned downstream of the GST coding sequence and TEV protease site in the pFN2A (Promega) *Escherichia coli* expression vector. The coding sequence was found to be identical to the GenBank™ sequence (NM_007726.3). Cells expressing the KRX/pFN2A-CNR1 (C-terminus), KRX/pFN2A-NR1 (segments C0-C2), NR1 (segments C0-C1-C2), and KRX/pFN2A-HINT1 plasmids were obtained as previously described (58, 59). The proteins were purified under native conditions on GStrap FF columns (GE Healthcare; 17-5130-01), and the fusion proteins retained were cleaved on the column with ProTEV protease (Promega; #V605A), collected, and concentrated in a centrifugal filter device (10,000 nominal molecular weight limit, Amicon Microcon YM-10 #42407; Millipore). The TEV protease was removed by immobilization on affinity resins (Amersham Biosciences; #17-0575-01).

The interactions between the NR1 C terminal sequences C0-C1-C2 (100 nM) and C0-C2 (100 nM), the CNR1 C terminus (100 nM), and the HINT1 protein (200 nM) were studied. Having demonstrated that the NR1 or HINT1 proteins did not bind to GST (100 nM: Genscript; USA Z02039; negative

control), GST-CNR1 and GST-NR1 sequences were used to study their mutual interactions and of those with HINT1. Media containing 450 μ l of HBS-EP buffer (10 mM HEPES [pH 7.4]; 150 mM NaCl; 3 mM EDTA; 0.005% P20) was mixed by rotation for 30 min at room temperature, after which glutathione-Sepharose was added to the protein mixture. The pellets obtained by centrifugation were washed thrice and solubilized in 2 $\times$ Laemmli buffer. The presence of NR1 C-terminal sequences was assessed in western blots probed with an antibody against the C2 segment.

BiFC analysis

Full-length murine NR1 (C0–C1–C2), HINT1, and CNR1 were sub-cloned in-frame into the pCE-BiFC-VN173 and pCE-BiFC-VC155 plasmids using standard cloning strategies (58, 59). CHO cells were transfected using Lipofectamine 2000 (Invitrogen) and incubated for 24 h before testing for transgenic expression.

SPR analysis

Interactions were determined using a BIACORE X (GE) coupling NR1 C-terminal sequences C0–C1–C2 or C0–C2 and the CNR1 C terminal sequence (50 μ g/ml) to Channel-2 of CM5 sensor chips by amine coupling at pH 7.0, with channel-1 acting as the blank. The sensor surface was equilibrated with HBS-EP buffer (GE; BR-1001-88) and after passing the CNR1-Ct or HINT1 (75 μ l) over the sensor surface, the sensorgrams were collected at 25°C with a flow rate of 5 μ l/min. Increasing analyte concentrations were studied, and the results were plotted with the BIAevaluation software (v 4.1).

Primary neuronal cultures, measurement of cell death, and immunocytochemistry

Neuron-enriched mouse cerebral cortical cultures were prepared from the brains of embryonic day-16 wild-type 129 and HINT1 knockout mice. The cerebral cortex was dissociated and seeded (1.25×10^5 cells/cm²) onto multiwell dishes coated with poly-D-lysine. After 3 h, the culture medium was changed to Neurobasal medium supplemented with B-27, GlutaMAX, and antibiotics (100 IU/ml Penicillin and 100 μ g/ml Streptomycin). From days 5 to 7 *in vitro*, cytosine arabinoside (5 μ M) was added to the cultures to eliminate the majority of proliferating non-neuronal cells. Cultures were maintained at 37°C in a humidified 5% CO₂ incubator.

Between days 12 and 14 *in vitro*, the cultures were rinsed with serum-free minimal essential medium and treated for 24 h with NMDA, with or without WIN55,212-2. Cell death was quantified by measuring lactate dehydrogenase (LDH; Roche) release into the bathing medium over 24 h, and it was expressed as the percentage of cell death induced by a maximal cytotoxic concentration (500 μ M) of NMDA: $(\text{LDH} - \text{LDH}_{\text{control}})/(\text{LDH}_{\text{NMDA}} - \text{LDH}_{\text{control}}) \times 100\%$.

Cells plated onto poly-D-lysine coated 10 mm glass coverslips were fixed for 10 min in 4% paraformaldehyde, incubated in 10% normal goat serum and 0.1% Triton X-100 in phosphate buffer saline. The cells were immunolabeled for 2 h at room temperature with an antibody against MAP2 (M1406; Sigma-Aldrich). The cells were then incubated with Alexa fluor 488 or 594-conjugated secondary antibodies (Invitrogen) and, finally, with 4',6-diamidino-2-phenylindole (DAPI), be-

fore mounting in Mowiol solution (Calbiochem). The coverslips were observed with a Leica DMI 6000 inverted fluorescence microscope (Leica Microsystemas S.L.U.). Controls were performed to confirm the specificity of the primary and secondary antibodies.

Calcium imaging

Primary cultures for calcium imaging were similar to those used to measure cell survival with the exception that the dissociated cells were grown on glass coverslips. Before testing, the coverslips were removed from the incubator and for calcium imaging, the medium was artificial cerebrospinal fluid containing mM NaCl (125), NaHCO₃ (20), KCl (5), MgCl₂ (2), CaCl₂ (2.5), glucose (10), and HEPES (20). The cells were loaded with Oregon Green[®] 488 BAPTA-1, AM (Invitrogen; O-6807) *via* immersion in medium. Before exposure to NMDA (15 μ M), cells were pretreated with cannabinoids through a glass pipette placed visually in the immediate vicinity of a selected cell to be imaged. Changes in intracellular calcium concentration were reflected by changes in the fluorescence ratio after excitation at 494/523 nm. Actual intracellular calcium concentrations could not be calculated in this study; however, relative increases in the fluorescence ratio indicated an increased [Ca²⁺]_i concentration within the cell (25, 69). After NMDA delivery, this fluorescence ratio increased to a maximum within 200 ms and then, rapidly returned to baseline. Fluorescence ratios obtained when measured 2 min after exposure to NMDA were normalized to the resting baseline (no NMDA) and then averaged across 10–15 different cells for a comparison between treatment conditions.

Statistical significance

ANOVA was followed by the Student–Newman–Keuls test (SigmaStat; SPSS Science Software), and significance was defined as $p < 0.05$.

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Author Disclosure Statement

The authors declare that, excluding income received from their primary employer “Ministerio de Economía y Competitividad (MINECO),” no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional services and that there are no personal financial holdings which could be perceived as constituting a potential conflict of interest.

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Abbreviations Used

Akt = protein kinase B
 BiFC = bimolecular fluorescence complementation
 CaMKII = Ca²⁺/calmodulin-dependent protein kinase II
 CCI = chronic constriction injury
 Chelerythrine = 1,2-Dimethoxy-N-methyl(1,3)-benzodioxolo(5,6-c)phenanthridinium chloride

CHO = Chinese hamster ovary
 CNR1 = cannabinoid receptors type 1
 CNS = central nervous system
 CP55,940 = (–)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol
 D-AP5 = D-(–)-2-Amino-5-phosphonopentanoic acid
 DPAT = (±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide
 GPCR = G protein coupled receptor
 HINT1 = histidine triad nucleotide-binding protein 1
 icv = intracerebroventricular
 Ifenprodil = α -(4-Hydroxyphenyl)- β -methyl-4-benzyl-1-piperidineethanol (+)
 JE907 = *N*-(1,3-Benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2-oxo-8-(pentyloxy)-3-quinolinecarboxamide
 KN62 = (*S*)-5-Isoquinolinesulfonic acid 4-[2-[(5-isoquinoliny)sulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)-propyl]phenyl ester, 1-[*N*,*O*-bis(5-Isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine
 KN93 = *N*-[2-[*N*-(4-Chlorocinnamyl)-*N*-methylaminomethyl]phenyl]-*N*-(2-hydroxyethyl)-4-methoxybenzene-sulfonamide phosphate salt
 LDH = lactate dehydrogenase
 L-NNA = NG-Nitro-L-arginine
 LY320135 = 4-[[6-Methoxy-2-(4-methoxyphenyl)-3-benzofuranyl] carbonyl] benzonitrile
 MK 801 = Dizocilpine/(5*S*,10*R*)-(+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a,d*] cyclohepten-5,10-imine maleate
 NMDA = *N*-methyl-D-aspartate
 NMDAR = *N*-methyl-D-aspartate receptor
 nNOS = neural nitric oxide synthase
 NO = nitric oxide
 PKA = protein kinase A
 PKC = protein kinase C
 RGSZ2 = regulator of G protein signaling 17 (Z2)
 ROS = reactive oxygen species
 SPR = surface plasmon resonance
 Win55,212-2 = (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate
 WT = wild type