International Journal of Neuropsychopharmacology (2014), **17**, 1943–1955. © CINP 2014
doi:10.1017/S1461145714000029

ARTICLE

The calcium-sensitive Sigma-1 receptor prevents cannabinoids from provoking glutamate NMDA receptor hypofunction: implications in antinociception and psychotic diseases

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Abstract

Through the cannabinoid receptor 1 (CB1), the endocannabinoid system plays a physiological role in maintaining the activity of glutamate *N*-methyl-D-aspartate (NMDA) receptor within harmless limits. The influence of cannabinoids must be proportional to the stimulus in order to prevent NMDAR overactivation or exaggerated hypofunction that may precipitate symptoms of psychosis. In this framework, the recently reported association of CB1s with NMDARs, which mediates the reduction of cannabinoid analgesia promoted by NMDAR antagonism, could also support the precipitation of schizophrenia brought about by the abuse of smoked cannabis, mostly among vulnerable individuals. Accordingly, we have investigated this possibility using neuroprotection and analgesia as reporters of the CB1–NMDAR connection. We found that the Sigma 1 receptor (σ1R) acts as a safety switch, releasing NMDARs from the influence of CB1s and thereby avoiding glutamate hypofunction. In $\sigma 1R^{-/-}$ mice the activity of NMDARs increases and cannot be regulated by cannabinoids, and NMDAR antagonism produces no effect on cannabinoid analgesia. In wild-type mice, ligands of the σ1R did not affect the CB1-NMDAR regulatory association, however, experimental NMDAR hypofunction enabled σ1R antagonists to release NMDARs from the negative control of CB1s. Of the σ1R antagonists tested, their order of activity was: S1RA > BD1047 ≫ NE100 = BD1063, although SKF10047, PRE-084 and (+)pentazocine were inactive yet able to abolish the effect of S1RA in this paradigm. Thus, the σ1R controls the extent of CB1-NMDAR interaction and its failure might constitute a vulnerability factor for cannabis abuse, potentially precipitating schizophrenia that might otherwise be induced later in time by the endogenous system.

Received 22 October 2013; Reviewed 13 November 2013; Revised 17 December 2013; Accepted 6 January 2014; First published online 31 January 2014

Key words: Antinociception, cannabinoid receptor type 1, HINT1 protein, NMDA receptor hypofunction, schizophrenia, Sigma-1 receptor.

Introduction

As the schizophrenia phenotype has gradually become better characterized, the idea of neurodevelopmental dysfunction as the exclusive cause of this mental disorder has lost steam (Broome et al., 2005). Although, the alterations underlying this disorder remain ill-defined in hierarchical terms, it has consistently been found that relevant genes interact closely with glutamatergic systems in general and, more specifically, with the activity of the *N*-methyl-D-aspartate receptor (NMDAR) (Kristiansen et al., 2007; Moghaddam and Javitt, 2012). Indeed, the data available suggest that NMDAR hypofunction leads to the dopaminergic deregulation observed in the striatal and prefrontal regions of schizophrenic patients (Harrison and Weinberger, 2005; Javitt, 2007), and that both these abnormalities underlie the symptoms recognized as schizophrenia (Mohn et al., 1999).

NMDAR hypofunction may be the result of deficient stimulatory regulation, alterations in the expression or structure of the receptor itself, and/or de-regulation of receptor signalling (Mohn et al., 1999; Labrie and Roder, 2010). In this context, cannabinoid-induced psychosis would most likely reflect the latter. One physiological role of the endocannabinoid system is to restrain NMDAR activity, maintaining it within safe limits and thereby protecting neural cells against excitotoxicity. It is the activity of the NMDARs themselves that makes the demands on the endogenous cannabinoid system in order to control their calcium currents (Marsicano et al., 2003). Thus, the cannabinoid system must equilibrate its negative influence to the strength of the NMDAR signals.

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Should this balance be disrupted, the lack of harmonization could provoke NMDAR-mediated cell damage or excessive down-regulation of glutamate signalling that would negatively affect cell homeostasis. Indeed, exogenous cannabinoids acting at the wrong time or exerting an inappropriate influence on their receptors could also cause NMDAR hypofunction (Javitt, 2007).

Despite the initial controversy, there is now a large body of evidence showing an association between cannabis abuse and psychosis (Arseneault et al., 2004). Although early exposure to cannabis can lead to psychosis and it circumstantially precipitates or intensifies the symptoms of schizophrenia (Degenhardt et al., 2003), this is only observed in certain subsets of vulnerable individuals (Cannon and Clarke, 2005). Therefore, NMDAR activity could be regulated by molecular mechanisms that prevent its hypofunction and should such failsafe mechanisms be disrupted, they could constitute factors of vulnerability when exogenous substances like cannabinoids push this system beyond its limits.

The cannabinoid receptor primarily implicated in NMDAR regulation is the type 1 receptor (CB1) (Marsicano et al., 2003; Liu et al., 2009). Whilst, the CB1 is found mostly in the pre-synapse, it is also found associated with NMDARs and the PSD95 protein in the post-synapse (Marchalant et al., 2008; Sánchez-Blázquez et al., 2013). Post-synaptic CB1s restrain NMDAR function by interfering with its signalling (Liu et al., 2009; Hampson et al., 2011), as well as through a direct interaction with the calcium channel (Liu et al., 2009; Li et al., 2010). A recent study demonstrated a direct physical interaction between CB1s and the NMDAR NR1 subunits, and the presence of both these receptors in functional protein ensembles along with the histidine triad nucleotide-binding protein 1 (HINT1). Thus, CB1s are recruited by activated NMDARs, where cannabinoids can serve to diminish calcium fluxes by driving the co-internalization of CB1s and NR1 subunits (Sánchez-Blázquez et al., 2013; Vicente-Sánchez et al., 2013).

It has to date been difficult to identify factors associated with susceptibility to the precipitation of schizophrenia by cannabis and thus, the existence of functional CB1-NMDAR protein assembles and their physiological regulation offers a new perspective to analyse such dysfunctions. This association seems to account for the protection against NMDAR-mediated excitotoxicity offered by cannabinoids (Vicente-Sánchez et al., 2013), it also influences the control of noxious stimuli perception during glutamatergic activation (Richardson et al., 1998) and it mediates the reduction of cannabinoid analgesia produced by NMDAR antagonism (Sánchez-Blázquez et al., 2013). In this scenario, glutamate activation brings NMDARs under the control of CB1s, a process that requires a molecular linchpin that is probably triggered by local increases in NMDAR calcium fluxes. This role is assumed by the CB1-bound HINT1 protein, which is recruited to NR1 subunits in response to increases in NMDAR-CaMKII activation (Sánchez-Blázquez et al., 2013; Vicente-Sánchez et al., 2013). However, the calcium-sensor breaker that disconnects NMDARs from the inhibition caused by cannabinoids remains unknown. In this respect, the Sigma 1 receptor (σ1R) has emerged as a potential candidate as its interaction with other proteins is promoted by calcium (Hayashi and Su, 2007; Ortega-Roldan et al., 2013), and it associates with NMDARs and regulates calcium influx (Monnet et al., 1990; Kume et al., 2002). Accordingly, it is feasible that σ1R prevents NMDAR hypofunction and its dysfunction could constitute a risk factor for psychosis in association with cannabinoid abuse.

In order to address this interesting issue, neuroprotection and antinociception were used to study the role of σ1R system in the stability of the CB1-NMDAR connection. We found that the σ1R sustains the CB1-NMDAR association, making the control exerted by cannabinoids over NMDARs more efficient and importantly, σ1R antagonists disrupt this association during NMDAR hypofunction to recover normal NMDAR activity.

Method

Animals

Wild type and homozygous ($\sigma 1R^{-/-}$) male sigma receptor knockout mice that were backcrossed (N10 generation) onto a CD1 albino genetic background (Harlan Ibérica, Barcelona, Spain) were used in this study. Null mutant mice were generated by targeted removal of most of the coding region of the *mSR1* gene, as described previously (Langa et al., 2003). The mice were housed and used in strict accordance with the European Community guidelines for the Care and Use of Laboratory Animals (Council Directive 86/609/EEC). The Committee on Animal Care at CSIC approved all the procedures for the handling and sacrificing animals.

Drugs, intracerebroventricular injection, and evaluation of antinociception

WIN55,212-2 (#1038), ACEA (#1319), methanandamide (#1782), NMDA (#0114), MK801 (#0924), Ifenprodil (#0545), D-AP5 (#0106), BD1063 (#0883), BD1047 (#0956), PRE084 (#0589), NE100 (#3133), SKF10047 (#1079) were all obtained from Tocris Bioscience (UK). We also used BD1063 (EST0013430.A; 1-[2-(3,4-dichlorophenyl) ethyl]-4-methylpiperazine dihydro- chloride), (+) Pentazocine (EST0064174) and the newly synthesized S1R antagonist S1RA (EST-52862.A; 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine (Diaz et al., 2012) provided by Laboratorios Esteve. The compounds were each injected into the lateral ventricle of mice in a volume of 4 μl as described previously (Haley and McCormick, 1957). The response of the animals to nociceptive stimuli was assessed using the warm water (52 °C) tail-flick test. Baseline latencies

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ranged from 1.7 to 2.0 s and they were not significantly affected by the σ1R ligands, NMDAR antagonists or the solvent used: saline, 1.8 ± 0.2 s; and ethanol/cremophor EL/physiologic saline (1:1:18), 1.9 ± 0.2 s ($n = 10$). A cut-off time of 10 s was used to minimize the risk of tissue damage. Antinociception is expressed as a percentage of the maximum possible effect (MPE = 100 × [test latency-baseline latency]/[cut-off time-baseline latency]). Groups of 8–10 mice received a dose of cannabinoid agonist and antinociception was assessed at different time intervals thereafter.

Primary cortical cell culture and measurement of cell death

Neuron-enriched mouse cerebral cortical cultures were prepared from the brains of embryonic day (E)16 CD1 mice. Cerebral cortices were dissociated and seeded (1.25×10^5 cells/cm^2) in multi-well 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 μg/ml Streptomycin solution: Invitrogen, UK). From day 5–7 *in vitro*, cytosine arabinoside (5 μM) was added to the cultures to eliminate the majority of proliferating non-neuronal cells and the cultures were maintained at 37 °C in a humidified 5% CO_2 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, in the presence or absence of the other drugs. Cell death was quantified by measuring lactate dehydrogenase (LDH, Roche) release into the bathing medium over 24 h and it was expressed as a percentage of the cell death induced by a maximal cytotoxic concentration (500 μM) of NMDA: (LDH – LDHcontrol)/(LDHNMDA – LDHcontrol) × 100%.

In vitro *interactions between recombinant proteins*

The interaction of HINT1 (200 nM) with either the NR1 C-terminal sequence C0–C1–C2 (100 nM) or σ1R (100 nM) was studied. Bait proteins were immobilized by covalent attachment to NHS-activated sepharose 4 fast flow (GE # 17-0906-01). The HINT1 protein was incubated with the sepharose, either alone (negative control) or together with the immobilized proteins, in a volume of 400 μl (50 mM Tris-HCl pH 7.4 and 0.2% CHAPS), for 40 min at room temperature (RT) with rotation. The pellets obtained by centrifugation were washed three times, solubilized in 2 × Laemmli buffer and analysed in Western blots. The interaction between the NR1 C-terminal sequence C0–C1–C2 and the σ1R was also studied. Moreover, the influence of Ca^{2+} on this association was evaluated. Incubations of recombinant proteins were carried out in the presence of 2.5 mM $CaCl_2$ and after 40 min, the pellets were processed as described above.

In vitro *competition binding assay*

The possible mutual interference of HINT1 and σ1R in terms of their binding to NR1 C-terminal sequence C0-C1-C2 was assessed. The recombinant HINT1 (200 nM) was incubated with immobilized NR1 C0-C1-C2 (100 nM) in the presence of increasing amounts of σ1R (100 nM, 300 nM) for 40 min at RT in a buffer containing 50 mM Tris-HCl pH 7.4, 2.5 mM $CaCl_2$ and 0.2% CHAPS. After incubation, the pellets obtained by centrifugation were washed three times, solubilized in 2 × Laemmli buffer and analysed in Western blots.

Immunoprecipitation and Western blotting

The preparation of membrane and cytosolic fractions, and the immunoprecipitation from brain synaptosomes of CB1s and NR1 subunits, was performed as described previously (Garzón et al., 2005; Rodríguez-Muñoz et al., 2007). The specificity and efficacy of the antibodies used in immunoprecipitation assays have been addressed elsewhere (Garzón et al., 2009; Sánchez-Blázquez et al., 2013). The immunocomplexes recovered were resolved by SDS/polyacrylamide gel electrophoresis (PAGE) and the separated proteins were then transferred onto 0.2 μM polyvinylidene difluoride (PVDF) membranes (BioRad 162-0176). The membranes were probed with the primary antibodies and their binding was detected using secondary antibodies conjugated to horseradish peroxidase. Antibody binding was visualized by chemiluminescence (GE Healthcare-Amersham, UK; ECL Prime WBDR, RPN2232) and recorded with a ChemiImager IS-5500 (Alpha Innotech, USA). Densitometry was performed using Quantity One Software (BioRad) and expressed as the mean ± S.E.M 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 typically repeated three times on samples derived from independent groups of mice and the results were always comparable. Equal loading was verified and the results adjusted if necessary to the quantities of actin or the immunoprecipitated housekeeper protein.

Bimolecular fluorescence complementation (BiFC) analysis

The plasmid pPD49.83 was used to generate two cloning vectors for BiFC analysis (generously provided by Dr Chang-Deng Hu at Purdue University, USA). Full-length murine NR1 (C0–C1–C2), CB1 and σ1R were sub-cloned in-frame into the pCE-BiFC-VN173 and pCE-BiFC-VC155 plasmids using standard cloning strategies (Rodríguez-Muñoz et al., 2011). Chinese hamster ovary (CHO) cells were transfected using Lipofectamine 2000 (Invitrogen) and incubated for 24 h prior to testing for transgene expression. Samples were visualized by confocal microscopy on glass bottom plates (MatTek Co,

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USA) using a Leica DMIII 6000 CS confocal fluorescence microscope equipped with a TCS SP5 scanning laser.

Statistical significance

Analysis of variance (ANOVA) tests were performed, followed by the Student Newman–Keuls test (SigmaStat, SPSS Science Software, Germany), and significance was defined as $p<0.05$.

Results

The σ1R drives cannabinoid negative control on NMDAR activity

While σ1Rs associate with NMDARs and control their calcium influx (Monnet et al., 1990; Kume et al., 2002), it is not known whether this receptor influences the capacity of cannabinoids to restrain NMDAR activity. Therefore, we addressed this issue through the neuroprotection that cannabinoids provide in a situation of NMDAR-mediated excitotoxicity. Cultured cortical neurons are suitable for this purpose because at this stage of development they express NMDARs, CB1s and σ1Rs (Nishikawa et al., 2000). Notably, NMDA increased cell death of $\sigma1R^{-/-}$ neurons and whilst WIN55,212-2 protected $\sigma1R^{+/+}$ neurons against a NMDA insult, this protection was not extended to $\sigma1R^{-/-}$ neurons (Fig. 1). Thus, the σ1R is essential for the negative control of NMDAR activity, in the presence or absence of activated CB1s.

Certain σ1R ligands such SKF10047 have been shown to produce neuroprotection by inhibiting NMDAR activity (Lockhart et al., 1995) and therefore, we determined whether the σ1R ligands studied displayed such parallel activity. The σ1R agonists (PRE084 and (+)Pentazocine) or the σ1R antagonists (BD1047, BD1063, S1RA and NE100) did not alter the capacity of NMDA to promote excitotoxicity in wild-type or $\sigma1R^{-/-}$ neurons (data for the antagonist S1RA and the agonist PRE084 are shown in Fig. 1). As expected SKF10047 produced neuroprotection in this experimental model of excitotoxicity; however, this activity was also present in cells devoid of the σ1R, indicating that this compound binds to and diminishes NMDAR activity. On the other hand, the selective σ1R antagonist S1RA did not alter the capacity of WIN55,212-2 to protect wild-type neurons against NMDAR-mediated excitotoxicity. Thus, in this paradigm the binding of agonists or antagonists to the σ1R does not increase NMDAR activity or disconnect this glutamate receptor from CB1 control, phenomena observed in $\sigma1R^{-/-}$ cultured neurons for WIN55,212-2 and NMDA insult. Rather, the presence of the σ1R dampens NMDAR activity and it also positions the NMDAR under the negative control of the CB1. Obviously, release of NMDARs from such regulatory association enhances their response to activation.

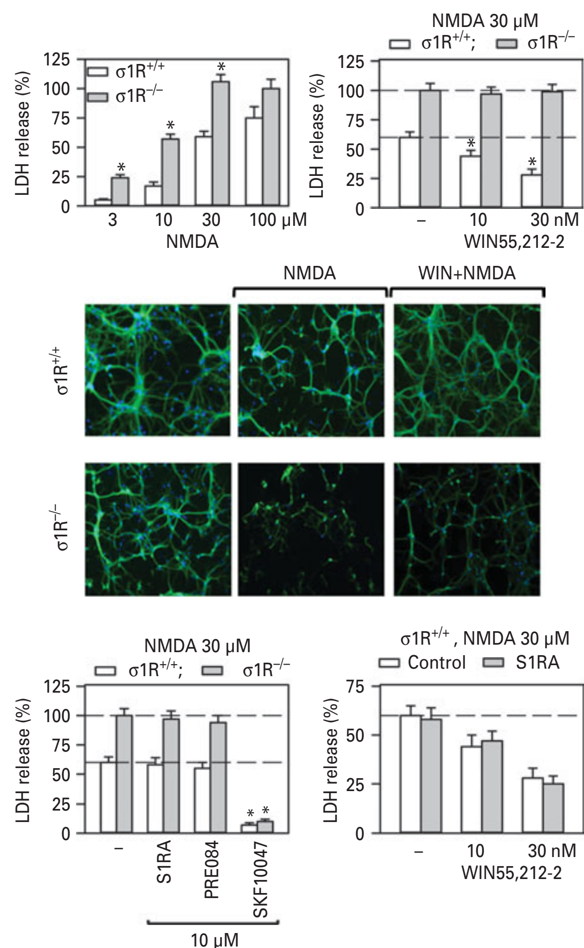

Fig. 1. *The absence of σ1Rs enhances NMDAR activity and compromises CB1-mediated neuroprotection against excitotoxicity.* Upper left panel: cortical cell cultures from wild-type ($\sigma1R^{+/+}$) and $\sigma1R^{-/-}$ mice were exposed to increasing concentrations of NMDA for 24 h. The LDH efflux into the medium measured cell death. The data shown are the mean±S.E.M from 20 wells per group. *Significant difference between wild-type and $\sigma1R^{-/-}$ cultured neurons (ANOVA/Student Newman–Keuls test, $p<0.05$, Sigmaplot, v12.5, SPSS Science Software, Germany). Right panel: cultures were exposed to a fixed concentration of 30 μM NMDA for 24 h in the presence or absence (–) of increasing concentrations of the cannabinoid agonist WIN55,212-2. *Significant difference with respect to NMDA alone (ANOVA/Student Newman–Keuls test, $p<0.05$). Middle panel: fluorescence photomicrographs of cortical cell cultures immunolabelled with an anti-MAP2ab. First row, from left to right: wild-type, wild-type treated with 30 μM NMDA, wild-type treated with 30 μM NMDA plus 30 nM WIN55,212-2. Second row, from left to right: $\sigma1R^{-/-}$, $\sigma1R^{-/-}$ treated with 30 μM NMDA, $\sigma1R^{-/-}$ treated with 30 μM NMDA plus 30 nM WIN55,212-2. Lower left panel: effect of σ1R agonists PRE084 and SKF10047, and of the σ1R antagonist S1RA, on the viability of cortical cell cultures from wild-type and $\sigma1R^{-/-}$ mice exposed to 30 μM NMDA for 24 h. *Significant difference with respect to the corresponding group receiving NMDA alone (ANOVA/Student Newman–Keuls test, $p<0.05$). Lower right panel: effect of σ1R antagonist S1RA (10 μM) on NMDA excitotoxicity and the protection of cell viability that WIN55,212-2 produces in this paradigm.

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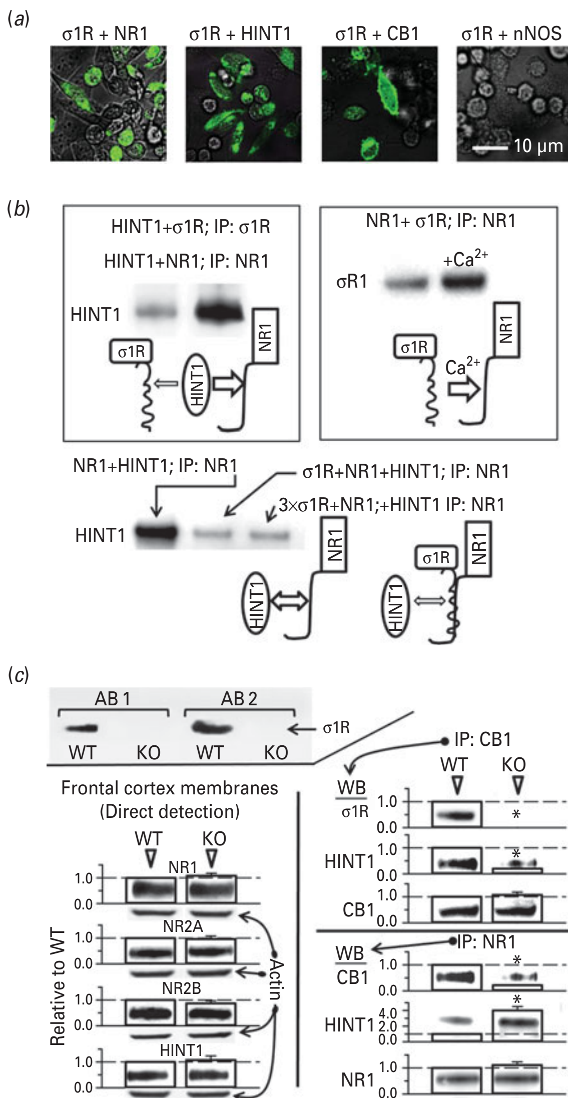

Fig. 2. *Interactions of σ1Rs with CB1s, NMDAR NR1 subunits and HINT1 proteins*. (*a*) The σ1R physically interacts with NMDAR NR1 subunits, HINT1 proteins and CB1s. Visualization of these interactions by bimolecular fluorescence complementation (BiFC). CHO cells were transiently co-transfected with cDNAs encoding the pair of proteins of interests in VN173 and VC155 (0.3 µg), and confocal fluorescent signals were obtained when VN173 and VC155 associated. Scale bar 10 µm. (*b*) *In vitro* association between the σ1R and NR1 C0-C1-C2 with the HINT1 protein. As HINT1 proteins form dimers, the protomer was used at 200 nM, whereas NR1 C-terminal sequence C0-C1-C2 and σ1R were used at 100 nM. Bait proteins (NR1 C0-C1-C2 and σ1R) were immobilized by covalent attachment to NHS-activated sepharose and the prey proteins alone did not bind to the NHS-sepharose (negative control). After incubation, the proteins were resolved by SDS-PAGE chromatography and analysed in Western blots (WB). A similar study was carried out to evaluate the influence of calcium on the association between NR1 C0-C1-C2 and σ1R. Competition experiments were conducted to study the possible mutual interference of HINT1 and σ1R for binding to the NR1 C-terminal sequence C0-C1-C2. The assays were repeated twice and the results were comparable. (*c*) Immunodetection of σ1R-related signals in the cerebral cortex of wild-type (WT) and σ1R$^{-/-}$ mice. Anti σ1R antibodies: AB1 (Invitrogen #423300, Camarillo, CA), AB2 (directed to 59–72 sequence, GenScript, Piscataway, NJ). The levels of signalling proteins related to NMDAR and CB1 activity in WT and σ1R$^{-/-}$ mice are shown. Mice were sacrificed and synaptosomes obtained from the cerebral cortex were processed to obtain the membrane (P2 fraction: see Method). The CB1s or NMDAR NR1 subunits were immunoprecipitated (IP) and the co-immunoprecipitated proteins were assessed in WB. Equal loading was verified and where necessary, the data from direct detection assays were adjusted using the actin signals. As the presence of CB1s and of NR1 subunits in WT and σ1R$^{-/-}$ mice was comparable, then these signals were used as the loading control for the co-immunoprecipitated proteins. The experiments were repeated three times using membranes from different groups of mice. Antibody binding was visualized through chemiluminescence signals with the ChemiImager IS-5500 system. Densitometry was determined using Quantity One Software (BioRad) and is expressed as the means±S.E.M of the integrated volume (average optical density of the pixels within the object area/mm^2). *Significantly different from the immuno-signals of the WT group (ANOVA/Student Newman–Keuls test, $p<0.05$).

The σ1R regulates the assembly of the CB1-HINT1-NMDAR protein complex

We have seen that the CB1 C terminus interacts directly with the C1 segment of the NMDAR NR1 subunit and that the HINT1 protein binds with both domains (Sánchez-Blázquez et al., 2013). Thus, after determining the relevance of the σ1R in the functional connection between the CB1 and the NMDAR, we determined whether the σ1R physically interacts with the proteins in this complex. CHO cells were transfected with plasmids expressing σ1R, NR1 (C0-C1-C2), HINT1 or CB1, and in biomolecular fluorescence complementation (BiFC) assays it became clear that σ1R binds directly to these proteins but not to proteins like neural nitric oxide synthase (nNOS) (Fig. 2*a*), or PKCγ (data not shown). *In vitro* assays carried out with recombinant proteins provided valuable clues on the mechanism by which σ1R could regulate the CB1-NMDAR interaction. Thus, the HINT1 protein displayed high affinity for the NR1 subunit and much lower affinity for the σ1R. As described for it's binding to Hsp70 in the endoplasmic reticulum (ER), the σ1R bound to the NR1 subunit in a calcium-dependent manner, an observation that is consistent with a role as a calcium-dependent safe switch. More critically, the σ1R interferes with the binding of HINT1 to the NR1 subunit and indeed, in its presence the association of these proteins was almost abolished (Fig. 2*b*).

Thus, we evaluated *ex vivo* the influence of the targeted deletion of the *σ1R* gene in the association between CB1s and HINT1 proteins, and of that of these proteins with NMDARs. The absence of the σ1R did not alter the expression of NMDAR subunits, or of CB1 and HINT1 proteins. However, in σ1R$^{-/-}$ cortical cells the association

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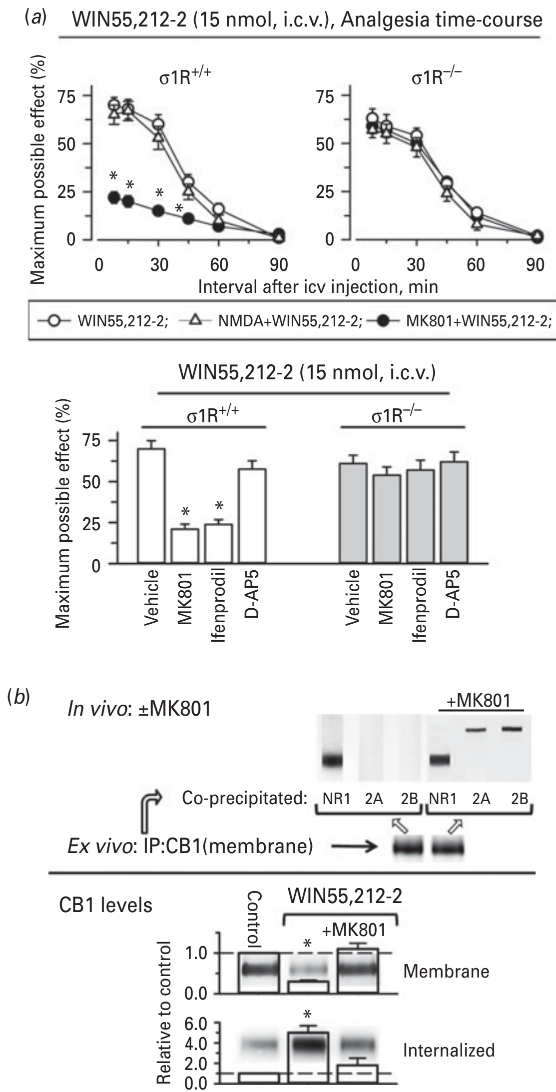

Fig. 3. *Antagonism of WIN55,212-2-induced supraspinal analgesia by MK801.* (*a*) Upper panel: The effect of 50 pmol NMDA, an activator of glutamate NMDARs, and of the NMDAR non-competitive antagonist MK801 (1 nmol) on the analgesia produced by 15 nmol WIN55,212-2 was evaluated in control (WT) and homozygous $\sigma 1R^{-/-}$ mice. *Significantly different from the control group that received WIN55,212-2 and saline instead of NMDA or the NMDAR antagonist, (ANOVA/ Student Newman–Keuls test, $p<0.05$). Lower panel: The activity of the NMDAR non-competitive antagonist Ifenprodil (3 nmol) and that of the competitive antagonist at the NMDAR glutamate-binding site, D-AP5 (3 nmol) was also evaluated. The NMDAR antagonists were injected i.c.v. 25 min before WIN55,212-2 (15 nmol) and analgesia was evaluated 15 min later in the warm-water (52 °C) tail-flick test. Each point or bar represents the mean±S.E.M of the data from 8 mice. *Significantly different from the group that received WIN55,212-2 and saline instead of the NMDAR antagonists, (ANOVA/Student Newman–Keuls test, $p<0.05$). (*b*) Upper panel: MK801 stabilizes the association between the NR1 and NR2 subunits of the NMDAR. The NMDAR antagonist MK801 (1 nmol, icv) was administered 30 min before euthanizing the mice. CB1 was immunoprecipitated from brain synaptosomes, and the co-immunoprecipitation of NR1 and NR2 subunits was then assessed in WB. MK801 did not alter CB1 content but it did recruit co-immunoprecipitated NR2 subunits. The assay was repeated twice on samples obtained from different mice and the results were comparable. Lower panel: WIN55,212-2 (15 nmol) was injected i.c.v. alone or together with MK801 (1 nmol), and the mice were sacrificed 3 h later. The CB1 was immunoprecipitated from the membrane and the soluble fraction of cortical synaptosomes, and its content determined. The experiments were repeated at least three times using membranes from different groups of mice. *Significantly different from the corresponding control value that did not receive the cannabinoid agonist (ANOVA/Student Newman–Keuls test, $p<0.05$). Details in Fig. 2*c*.

of the CB1 with HINT1 proteins and NMDAR NR1 subunits was impaired, whilst that of HINT1 with the NR1 subunit increased (Fig. 2*c*). Therefore, when bound to the NR1 subunit, the σ1R prevents the translocation of the HINT1 protein from the CB1 towards the NMDAR, thereby determining the quality of the CB1-HINT1-NR1 interaction.

Cannabinoid analgesia as a reporter of NMDAR hypofunction

The previous observations suggested that the mechanism by which σ1Rs regulate the functional interaction between CB1s and NMDARs could be calcium-dependent. Therefore, we looked for an assay in which NMDAR hypofunction could be easily identified in an *in vivo* assay, a situation that was provided by NMDAR antagonism greatly reducing calcium fluxes and causing significant reductions in the capacity of cannabinoid agonist to produce antinociception (Thorat and Bhargava, 1994; Sánchez-Blázquez et al., 2013). This feature was then used as reporter of experimental NMDAR hypofunction, which is also accepted as an animal model that reproduces certain aspects of schizophrenia observed in humans (Mouri et al., 2007).

While activation of NMDARs (i.c.v. injection of NMDA, 50 pmol) did not alter WIN55,212-2-evoked anti-nociception in wild-type mice, the non-competitive NMDAR antagonists MK801 (1 nmol/mouse) or Ifenprodil (3 nmol/mouse) strongly dampened WIN55,212-2 analgesia. Moreover, the competitive NMDAR antagonist D-AP5 produced a weaker effect in this paradigm, barely reaching the level of statistical significance (Fig. 3*a*). The molecular mechanisms underlying these effects seem to involve the association between CB1s and NR1 subunits, which can be extended to the whole ionotropic receptor due to the action of certain NMDAR antagonists. Whilst, MK801 and Ifenprodil bind simultaneously to the NR1 and NR2 subunits, to the pore-forming region (Kashiwagi et al., 2002) or their N-terminal sequences (Gallagher et al., 1996), respectively, competitive antagonists such as D-AP5 bind exclusively to the glutamate site in the NR2 subunit (Laube et al., 2004). Therefore, NMDAR antagonists such as

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MK801 or Ifenprodil stabilize the association between NR1 and NR2 subunits and the non-competitive nature of this interaction provides more durable NMDAR hypofunction than the competitive antagonists that can be displaced by endogenous regulators of this ionotropic receptor. In the presence of MK801 or Ifenprodil, cannabinoids hardly co-internalize the whole NMDAR together with the CB1 (Fig. 3*b*) and their analgesic effects diminish accordingly (Thorat and Bhargava, 1994; Sánchez-Blázquez et al., 2013).

It is particularly relevant that the NMDAR antagonists MK801 and Ifenprodil did not alter the capacity of cannabinoids to produce anti-nociception in $\sigma 1R^{-/-}$ mice, an observation that correlates with that made previously (Fig. 1), and that confirms the essential role of σ1Rs in the coupling of glutamate NMDARs with CB1s. Therefore, in wild-type mice normal cannabinoid analgesia reflects the permeation of calcium driven by the NMDARs. Notwithstanding, NMDAR antagonists, by reducing calcium influxes, produce NMDAR hypofunction and those that bind simultaneously to NR1 and NR2 subunits report this situation through reductions in cannabinoid antinociception.

We first characterized the analgesia produced by WIN55,212-2 administration (i.c.v.) and whether it could be altered by agonists or antagonists of the σ1R. This cannabinoid produced a dose-dependent anti-nociception in the tail-flick test, which was antagonized by the CB1 antagonist AM-251. Analgesia was induced rapidly, typically within 8–15 min post-injection, and it then slowly declined with no significant effect being detected 60 min after the highest doses were administered. The potency of WIN55,212-2 was similar in wild-type and $\sigma 1R^{-/-}$ mice, with both groups displaying comparable baselines (Supplementary Figure S1A). The anti-nociceptive effects of WIN55,212-2, ACEA or methanandamide in wild-type mice were not altered by i.c.v. administration of the σ1R antagonists S1RA, BD1063 and BD1047, or the agonist PRE084 (3 to 10 nmol per mouse: Supplementary Figure S1B). Similarly, these σ1R ligands did not alter cannabinoid analgesia in $\sigma 1R^{-/-}$ mice (data not shown).

NMDAR hypofunction enables antagonists of σ1R to disrupt the association of CB1 with NMDARs

The non-competitive NMDAR antagonist MK801 was used to address whether σ1R control of the CB1-NMDAR association was calcium-dependent. Notably, the ligands described as σ1R antagonists blocked the negative effect of MK801 and restored WIN55,212-2-evoked analgesia, while such activity was not displayed by σ1R agonists like PRE084, SKF10047 and pentazocine. The 25 min interval between the administration of σ1R antagonists and MK801 re-established the capacity of WIN55,212-2 to produce analgesia, whereas a 90 min interval between treatments or simultaneous administration was less effective. The influence of σ1R antagonists was dose-dependent and a maximal effect in this paradigm was observed at about 3 nmol i.c.v. (Supplementary Figure S2). Therefore, to compare the activity of different σ1R ligands we selected an interval of 25 min and doses of 3 nmol that did not alter WIN55,212-2 analgesia *per se* (Supplementary Figures S1 & S2). The effectiveness of the σ1R ligands in inhibiting the effect of MK801 on WIN55,212-2 analgesia was determined using the area under the curve over the post-WIN55,212-2 intervals of 10–60 min (Sigmaplot v12.5, Germany). The value that corresponded to diminishing effect of MK801 on WIN55,212-2 analgesia was subtracted from the analgesia produced by the combination of each σ1R ligand with WIN55,212-2 and the ratios of the resulting values were computed. In this assay, S1RA was the most potent ligand in terms of favouring WIN55,212-2 analgesia against MK801 diminishing effects. As such, we attributed an arbitrary value of 1 to this antagonist and we established a ranking of: S1RA > BD1047 > NE100 = BD1063 ≫ (+) pentazocine = SKF10047 = PRE084 (Fig. 4*b*).

As this paradigm distinguished between antagonists and agonists, such regulation did appear to be mediated by σ1Rs. Indeed, as expected for σ1R-mediated regulation, the agonist PRE084 (6 nmol) blocked the effect of the selective σ1R antagonist S1RA, enabling MK801 to reduce WIN55,212-2-evoked analgesia (Fig. 5*a*). Similarly, the agonist SKF10047 (6 nmol) reduced the effect of S1RA activity (not shown). Whilst the σ1R antagonist S1RA was the most effective antagonist in this experimental model, other antagonists such as BD1047 achieved about 75% of its activity, while NE100 and BD1063 only reached approximately 50%. Thus, σ1R antagonist displayed partial effects and 10 nmol BD1063 or NE-100 reduced the activity of 3 nmol S1RA on MK801-WIN55,212-2 analgesia to the level observed for BD1063 when administered alone (Fig. 5*b*). Hence, some σ1R ligands would appear to behave as partial agonists in such demanding situations. Given that non-competitive antagonists like MK801 in this particular assay block NMDARs, σ1R ligands cannot produce effects by acting directly on the NMDAR (see e.g. SKF10047 in Fig. 1), and thus it might be useful to determine antagonist/agonist activities at the σ1R.

At the molecular level, $\sigma 1R^{-/-}$ neural cells display an enhanced NMDAR activity together with impairment in the association of the CB1-NMDAR complex and the HINT1 protein translocated to the NR1 subunit (Fig. 2*c*). Thus, these changes could prevent cannabinoid neuroprotection against NMDA insult, as well as NMDAR antagonism from altering CB1-mediated antinociception. By analogy, we considered the possibility that in wild-type mice NMDAR hypofunction allows σ1R antagonists to disrupt the CB1-NMDAR(MK801) complex, thereby restoring NMDAR activity. Indeed, the administration of S1RA before MK801 uncoupled CB1 from the NR1 and NR2 subunits, which had a positive effect on cannabinoid analgesia. In these circumstances and

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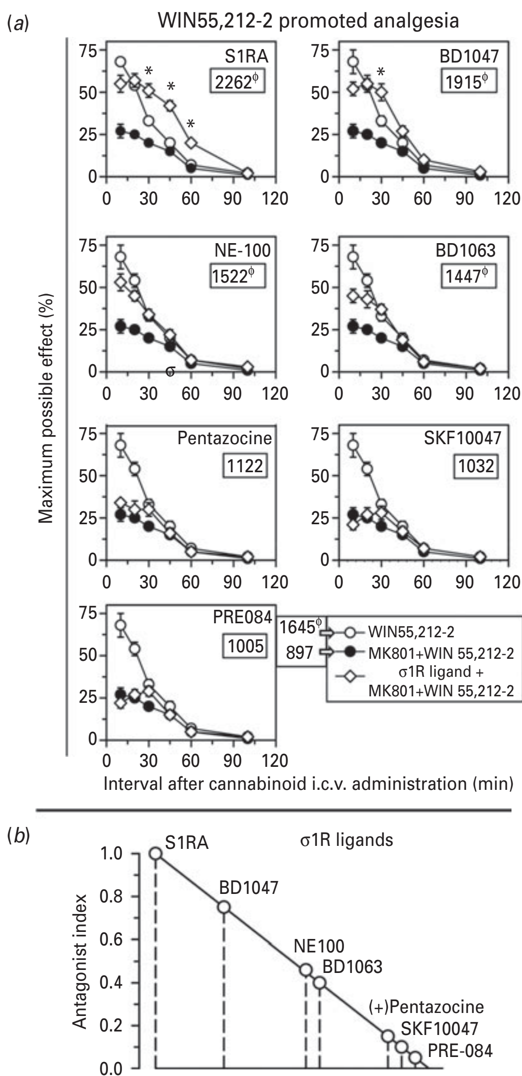

Fig. 4. *Antagonism of σ1Rs prevents the MK801 from reducing WIN55,212-2 analgesia*. (*a*) Control wild-type mice received a single i.c.v injection (3 nmol) of the σ1R antagonists S1RA, BD1047, NE-100 and BD1063, or of the σ1R agonists (+) Pentazocine, SKF10047 and PRE084 25 min before MK801 (1 nmol, i.c.v.). The CB1 agonist WIN55,212-2 (15 nmol, i.c.v.) was administered 25 min later and analgesia was evaluated at various time points. For each treatment in the study, groups of 16 mice were used. To separate consecutive evaluations of antinociception in the same animals, each group that had received an identical treatment was divided into two and antinociception was determined alternatively by the warm water (52 °C) tail-flick test. Thus, the values are the mean±S.E.M from groups of 8 mice. *Significantly different from the group that received WIN55,212-2 and the vehicle instead of the σ1R ligand and of MK801. The area under the curve computed in the post-WIN55,212-2 intervals 10–60 min is framed (Sigmaplot v12.5). Φ Indicates that at various intervals of the time-course the analgesic effects were statistically different to those of the MK801-WIN55,212-2 group (ANOVA/Student Newman–Keuls test, $p<0.05$). (*b*) Rank order of potency of the σ1R ligands to protect WIN55,212-2 analgesia from the reducing effect induced by MK801. Based on the data in (*a*), the area under the curve value corresponding to MK801-WIN55,212-2 was subtracted from the area under the curve value corresponding to σ1R ligand-MK801-WIN55,212-2. The antagonist index is expressed relative to the effect observed for the σ1R antagonist S1RA (given an arbitrary value of 1).

as observed for σ1R$^{-/-}$ mice, the HINT1 proteins moved from CB1s towards NMDAR NR1 subunits (Fig. 5*c*).

Discussion

This study indicates that the CB1 must cooperate with a series of signalling proteins to maintain the activity of NMDARs within physiological limits. We previously reported the essential role of the HINT1 protein to sustain such regulation (Sánchez-Blázquez et al., 2013; Vicente-Sánchez et al., 2013), and now, we reveal that the σ1R cooperates with HINT1 in this process. These two proteins are key elements in this regulatory pathway and the targeted deletion of either the *HINT1* or *σ1R* gene causes the NMDAR to uncouple from CB1 regulation, increasing glutamate receptor activity (Vicente-Sánchez et al., 2013; present study).

Sigma receptors are considered to be ligand-regulated molecular chaperones that have been implicated in diverse physiological processes, including addiction associated with alcohol and cocaine. Moreover, their de-regulation concurs with, or is related, to the pathophysiology of several neural diseases (Chen et al., 2007; Robson et al., 2012). Certain parallels exist between the σ1R when it is in the ER and with respect to its function at the cell plasma membrane (PM). Thus, σ1R binds to the nucleotide-binding domain of Hsp70 (ER) and NMDAR NR1 subunits (PM) in a calcium-dependent manner, and the depletion of calcium and ligand binding to σ1R disrupts both these associations (Hayashi and Su, 2007; Ortega-Roldan et al., 2013; present study). At the cell membrane, σ1R binds to ionotropic NMDARs and σ1R ligands modulate calcium influx through these receptors (Monnet et al., 1990; Shimazu et al., 2000). Our data suggests that σ1R ligands do not operate when activators such as NMDA enhance NMDAR activity but when cannabinoids produce excessive NMDAR hypofunction and reduce local calcium levels, and then σ1R antagonists disconnect both systems.

In terms of the molecular events involved, it appears that they follow an established sequence. Initially, the CB1 cytosolic C terminal sequence establishes direct interactions with the NR1-C1 segment, an association that is stabilized by the simultaneous binding of HINT1 to both receptors (Sánchez-Blázquez et al., 2013) and that dampens the NMDAR response to activation (Vicente-Sánchez et al., 2013). Appropriate calcium levels, probably maintained by the activity of the NMDARs, promote σ1R binding to the NR1-C1 segment. The NR1-C1 is essentially a positively charged domain, whereas HINT1

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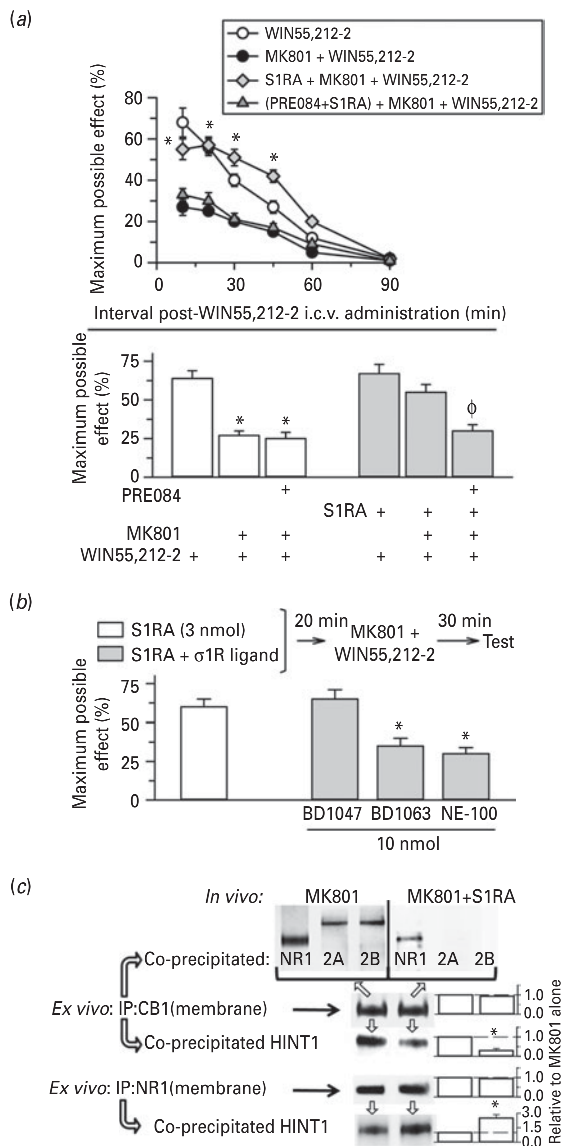

Fig. 5. *The effect of σ1R ligands on S1RA-induced rescue of cannabinoid analgesia from NMDAR antagonism.* (*a*) Upper panel: The analgesic effect of the CB1 agonist WIN55,212-2 (15 nmol, icv) was reduced in mice that received the NMDAR antagonist MK801 (1 nmol, −30 min, icv). In the absence of MK801 treatment, the σ1R antagonist S1RA or the agonist PRE084 did not alter WIN55,212-2 analgesia in wild-type mice. S1RA (3 nmol, icv) rescued WIN55,212-2 analgesia from the MK801 reducing effect, whereas PRE084 (6 nmol, icv) prevented S1RA from restoring cannabinoid analgesia in the MK801-treated mice. *Significantly different from the MK801 + WIN55,212-2 group. Lower panel: Data were obtained 15 min after WIN55,212-2 icv-injection. *Significantly different from the WIN55,212-2 control group; ϕ Significantly different from the S1RA-MK801-WIN55,212-2 group (ANOVA/Student-Newman-Keuls test, $p<0.05$). (*b*) The σ1R antagonists BD1047, BD1063 and NE-100 were used at 10 nmol in their interaction with 3 nmol of the σ1R antagonist S1RA. *Significantly different from the enhancing effect of S1RA on MK801-WIN55,212-2 control group (ANOVA/Student-Newman-Keuls test, $p<0.05$). Details in A. (*c*) The σ1R antagonist S1RA separates the HINT1-NMDAR (MK801) complex from the CB1. S1RA (3 nmol) or saline were administered 25 min before MK801 (1 nmol) and 25 min later, the mice were killed. The CB1 was immunoprecipitated from cortical synaptosomes, and the co-precipitation of NMDAR NR1/2 subunits and HINT1 proteins was determined in WB. NR1 was also immunoprecipitated to determine its association with HINT1 proteins. S1RA did not alter the presence of CB1s or of the NR1 subunits and thus, these signals were used as the loading control for the co-immunoprecipitated proteins. The experiments were repeated three times in different groups of mice. *Significantly different from the corresponding control value that received MK801 but not the σ1R antagonist S1RA (ANOVA/Student Newman–Keuls test, $p<0.05$). Details in Fig. 2*c*.

displays an ample negative charge (Protean, DNASTAR, Inc.) and σ1R reduces the avidity of the NR1-C1 subunit for the CB1-associated HINT1 protein (Fig. 6). It is the activity of the NMDAR that serves to recruit the CB1, probably through calcium-dependent kinases like CaMKII. In the presence of calcium, the σ1R antagonists do not disrupt the CB1-HINT1-NMDAR association and cannabinoids can promote the co-internalization of CB1-HINT1 complexes together with NR1 subunits, thereby reducing NMDAR activity (Sánchez-Blázquez et al., 2013).

If calcium diminishes beyond a critical level in the NMDAR environment, a series of concatenated changes would operate to physically and functionally uncouple NMDARs from the negative regulation of the CB1. As CaMKII activity requires calcium-calmodulin, the CaMKII-dependent formation of the CB1-HINT1-NR1 ensemble diminishes upon calcium depletion (Sánchez-Blázquez et al., 2013), and in the existing complexes, the antagonists can now remove the σ1Rs allowing NR1 subunits to bind freely with and remove the HINT1 proteins towards the NMDARs, irrespective of whether CB1 is activated or not. This situation might be comparable to what is observed in $\sigma1R^{-/-}$ mice, where cannabinoids display no control on NMDAR function. However, in wild-type mice increases in calcium levels restore the functional CB1-NMDAR connection by promoting σ1R binding to the NR1 subunit and the shift of the HINT1 protein back to the CB1. The segregation of the NMDAR-HINT1 complex from damping control of CB1 does not necessarily imply NMDAR de-regulation. The HINT1 protein when bound to the NR1 subunit, although in a much lesser extent than when coupled to the CB1-HINT1 complex, also limits NMDAR responsiveness to reduce the risk of excitotoxicity (Vicente-Sánchez et al., 2013).

Therefore, alterations in this calcium sensor would contribute to a de-regulated NMDAR activity. Indeed, its absence prevents the neuroprotective effects of endocannabinoids and since NMDAR activity increases in $CB1^{-/-}$ cortical cultured neurons that express HINT1 and σ1R proteins (Kim et al., 2006), this control is

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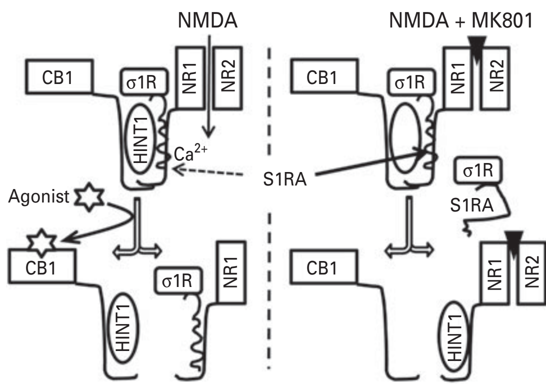

Fig. 6. *Diagram describing the role of HINT1 and σ1R in the CB1-NMDAR protein assembly.* Left Panel: The cytosolic C terminal sequence of CB1 binds to the NR1 subunit of the NMDAR, and HINT1 stabilizes this interaction (Sánchez-Blázquez et al., 2013). The NMDAR complexed to the non-activated CB1 permeates calcium, which stabilizes the binding of the σ1R to the NR1 subunit. In this scenario, antagonists of the σ1R, such as S1RA, do not alter the CB1-NR1 complex and cannabinoids activate the CB1 producing the co-internalization of the CB1-HINT1 along with the NR1 subunits, causing instability of the NMDAR and reducing its activity (Sánchez-Blázquez et al., 2013; Vicente-Sánchez et al., 2013). Right Panel: The reduction of calcium in the milieu of the σ1R-NR1 binding complex weakens the interaction between these proteins, enabling the antagonists to release σ1R from the CB1-NR1 complex. In the absence of σ1R, HINT1 probably binds to the NR1 regions previously covered by the σ1R and then translocates to the NR1 subunit. Thus, CB1 separates from the NR1-HINT1 complex, releasing NMDAR from the negative influence of cannabinoids. The experimental blockage of NMDARs with non-competitive antagonists or the use/abuse of exogenous cannabinoids would promote such decrease of calcium levels for σ1R antagonists to trigger this safe switch.

apparently not assumed by alternative systems. Thus, the CB1 emerges as a decisive negative physiological regulator of NMDAR function in the earliest development of cerebral cortical structures, albeit under the supervision of the σ1R system that is in charge of the plasticity and adaptation of this mechanism. Cannabinoids produce comparable analgesia in wild-type and $\sigma1R^{-/-}$ mice in which CB1 is disconnected from the NMDAR. Thus, the expression of cannabinoid analgesia does not require NMDAR activity and the relationship between these proteins in antinociception would mostly reflect their functional antagonism. Glutamate NMDAR activity is considered pro-nociceptive and cannabinoids oppose this function either producing NMDAR instability or antinociception. Indeed, experimental impairment of CB1 function provokes NMDAR-dependent allodynia and hyperalgesia (Richardson et al., 1998). More relevantly, in neuropathies where NMDAR hyperactivity diminishes the antinociceptive capacity of strong analgesics like opioids, cannabinoids may still display some of their analgesic effects (Ashton and Milligan, 2008).

In contrast to cannabinoids, opioids like morphine display greater analgesic effects in $\sigma1R^{-/-}$ mice (Sánchez-Fernández et al., 2013). Although the CB1 and the mu-opioid receptors (MOR) can associate with NMDARs (Rodríguez-Muñoz et al., 2012; Sánchez-Blázquez et al., 2013) these complexes are regulated differently. Whereas, activators of the NMDAR recruit the CB1 (Sánchez-Blázquez et al., 2013) as well as the activity of endocannabinoids (Marsicano et al., 2003), MOR-binding opioids promote the activation of the MOR-coupled NMDAR. The activated CB1 remains associated with the NMDAR to dampen calcium influx, but the activated MOR releases a functional NMDAR, which now stimulates negative feedback on opioid signalling (reviewed in Garzón et al., 2012). Thus, while cannabinoid analgesia is hardly influenced by the activity of NMDARs, opioid analgesia is definitively under their negative control (Pasternak et al., 1995). The differences between both systems also apply to the effects of σ1R ligands and whereas they do not significantly alter cannabinoid analgesia, σ1R antagonists clearly increase the potency of opioids to produce antinociception (Kim et al., 2010). This effect could derive from σ1R coupling the negative influence of NMDAR function on MOR signalling, thereafter opposing to opioid analgesia. Indeed, the absence of HINT1 proteins releases MORs from the negative control of NMDARs and morphine analgesia increases (Rodríguez-Muñoz et al., 2011). It is possible that in the MOR environment, σ1R antagonists prevent the recruitment of NMDARs, being permissive with MOR signalling. Further research is required to reveal the precise mechanism involved.

Alterations in the σ1R system have been consistently related to schizophrenia, and binding assays using post-mortem brains of schizophrenic patients demonstrated reductions in sigma binding sites in the occipital, frontal and temporal cortices, as well as in the cerebellum, and increases were also observed in the cingulate cortex (Weissman et al., 1991; Shibuya et al., 1992). These changes may be due to the presence of σ1Rs with diminished avidity for their ligands that will not adequately prevent cannabinoids from promoting NMDAR hypofunction. Clinical studies suggested that σ1R ligands might not possess anti-psychotic-like effects against positive symptoms but rather; they may ameliorate the negative symptoms that are mainly related to NMDAR dysfunction (Hayashi et al., 2011). Interestingly, the severity of the negative symptoms of schizophrenic patients is correlated with alterations in the plasma levels of neurosteroids, the putative ligands of this ligand-operated chaperone/receptor (Shirayama et al., 2002; Ritsner et al., 2007). Neurosteroids produce multiple effects on the nervous system, making it difficult to dissect out the processes mediated by σ1R. Nevertheless, pregnenolone levels are altered in post-mortem brains obtained from schizophrenia patients (Marx et al., 2006) and adjunct treatment with pregnenolone diminishes their negative

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symptoms (Marx et al., 2009; Ritsner et al., 2010). Collectively, these findings suggest that σ1R ligands may be useful in ameliorating specific symptoms of schizophrenia.

The potential anti-psychotic activity of σ1R ligands is believed to result from their antagonistic activity, although our data indicate that the term agonist or antagonist is not clear when considering the control of NMDAR hypofunction. The selective σ1R antagonist S1RA (Diaz et al., 2012; Romero et al., 2012) displayed the highest activity in this paradigm and its effect was counterbalanced by the agonist PRE084. However, several other σ1R ligands behaved as partial agonists that reduced the activity of S1RA in this context, such as NE100 and BD1063. The mixed agonist-antagonist activity of these compounds may also account for the complex σ1R pharmacology, which is further complicated by the neurosteroids that show multiple activities outside of this system.

Genetic alterations that cause a degree of NMDAR hypoactivity may collaborate with cannabinoids to produce psychotic symptoms (Di et al., 2012). However, those that affect the precise mechanism by which endogenous cannabinoids control NMDAR activity would mostly be identified as factors of vulnerability for psychosis brought about by cannabis abuse, in the context of which the *HINT1* and *σ1R gene* also appear to be relevant. In fact, the HINT1 protein has been associated with psychosis (Vawter et al., 2002, 2004) and there is evidence that associates variants of HINT1 with schizophrenia (Chen et al., 2008). On the other hand, the *σ1R* is a candidate gene to cause schizophrenia (Weissman et al., 1991; Shibuya et al., 1992; Watanabe et al., 2012), and the putative endogenous ligands of this receptor, neurosteroids like dehydroepiandrosterone (DHEA) and pregnenolone (PREG), as well as their sulfates, have been implicated in the pathophysiology of schizophrenia (Marx et al., 2009; Ritsner, 2011).

Thus, in the context of NMDAR regulation by CB1s, an anomalous σ1R, or deficits in its endogenous ligands, could maintain the functional connection between these receptors beyond the expected threshold. This anomaly would contribute to a disproportionate down-regulation of NMDAR activity (hypofunction), constituting a serious risk factor for the development of schizophrenia amongst cannabis abusers. While cannabis use in the general population does not affect the incidence of schizophrenia, triggering the influence of CB1 on NMDAR activity at the wrong time and frequency does reduce the age of onset of psychotic illness among vulnerable individuals, in whom the endogenous cannabinoid system would otherwise promote the onset of this condition at a slower rate.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145714000029.

Acknowledgments

This research was supported by MSC 'Plan de Drogas 2011–14' and the Ministerio de Economía y Competividad (MINECO), SAF 2012-34991. We would like to thank Concha Bailón and Gabriela de Alba for their excellent technical assistance. The authors acknowledge the assistance of Dr Mark Sefton (BiomedRed, SL) in the editing of this manuscript.

Statement of Interest

None

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