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ORIGINAL ARTICLE

A Cannabinoid Receptor-Mediated Mechanism Participates in the Neuroprotective Effects of Oleamide Against Excitotoxic Damage in Rat Brain Synaptosomes and Cortical Slices

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Abstract

A number of physiological responses in the central nervous system (CNS) are regulated by the endocannabinoid system (ECS). Inhibition of neuronal excitability via activation of cannabinoid receptors (CBr) constitutes a potential protective response against neurotoxic insults. Oleamide (ODA) is a fatty acid amide with endocannabinoid profile exerting several effects in the CNS, though its neuroprotective properties remain unknown. The tryptophan metabolite quinolinic acid (QUIN) elicits toxic effects via overactivation of *N*-methyl-D-aspartate receptors (NMDAr) after its accumulation in the CNS under pathological conditions. Here, we investigated the protective properties of ODA against the excitotoxic damage induced by QUIN in rat brain synaptosomes and cortical slices, and whether these effects are linked to the stimulation of the endocannabinoid system via CB1 and/or CB2 receptor activation. ODA (1–50 μM) prevented the QUIN (100 μM)-induced loss of mitochondrial reductive capacity in synaptosomes in a mechanism partially mediated by CB1 receptor, as evidenced by the recovery of mitochondrial dysfunction induced by co-incubation with the CB1 receptor antagonist/inverse agonist AM281 (1 μM). In cortical slices, ODA prevented the short-term QUIN-induced loss of cell viability and the cell damage in a partial CB1 and CB2 receptor-dependent manner. Altogether, these findings demonstrate the neuroprotective and modulatory properties of ODA in biological brain preparations exposed to excitotoxic insults and the partial role that the stimulation of CB1 and CB2 receptors exerts in these effects.

Keywords Excitotoxicity · Endocannabinoid system · Neuroprotection · Oleamide · Quinolinic acid · Cannabinoid receptors

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Introduction

Several toxic events contribute to the progression of neural cell death observed in different neurodegenerative disorders. Inflammatory events recruit compromised brain energy metabolism, oxidative stress, and excitotoxicity (Hensley et al. 2006; Hunter et al. 2007; Tilleux and Hermans 2007). The secretion and release of pro-inflammatory cytokines and neurotoxic metabolites by microglial cells and macrophages during the occurrence of inflammatory events in the brain is associated with the pathogenesis of several neurodegenerative disorders (Muhl and Pfeilschifter 2003; Aguilera et al. 2018). Inflammation-related stimulation of the kynurenine pathway (KP)—the catabolic route of tryptophan degradation—by activated microglia is responsible for increased levels of the intermediate excitatory metabolite quinolinic acid (2,3-pyridine-dicarboxylic acid or QUIN), thereby triggering

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excitotoxic events that contribute to the neurodegenerative patterns observed in neurological disorders with excitotoxic components, such as Parkinson's and Huntington's diseases (Schwarcz et al. 2012). QUIN exerts its toxic pattern mostly through the overactivation of *N*-methyl-D-aspartate receptors (NMDAr), subsequently increasing cytosolic Ca^{2+} concentrations and neuronal cell damage after triggering different toxic mechanisms, including free radical formation and oxidative damage, mitochondrial dysfunction, cytochrome c release, and ATP exhaustion (Pérez-De La Cruz et al. 2012; Ribeiro et al. 2006).

Together, endocannabinoids, enzymes for their synthesis and degradation, and plasmatic and mitochondrial (Bénard et al. 2012) membrane receptors (CB1 and CB2), comprise the endocannabinoid system (ECS), which is a complex system modulating neurotransmission in the brain. It has been demonstrated that the ECS stimulation via CB1 and/or CB2 receptor activation affords neuroprotective actions through several mechanisms, including reduction in the release of excitatory neurotransmitters at the presynaptic level (Nazari et al. 2016), the CB1 receptor coupling to NR1 subunits of the NMDAr via histidine triad nucleotide-binding protein 1 (HINT-1) to reduce excitatory transmission at the postsynaptic level (Sánchez-Blázquez et al. 2013; Sánchez-Blázquez et al. 2014; Rodríguez-Muñoz et al. 2016), and the negative modulation of the G protein-coupled chemokine receptor CXCR4 via a physical heterodimeric association with CB2 and further inhibition of noxious responses (Coke et al. 2016). In addition, cannabinoids can promote the activation of the peroxisome proliferator-activated receptors (PPARs) to reduce inflammatory responses at the nuclear level (O'Sullivan 2007; O'Sullivan 2016). Altogether, these mechanisms support the design of novel experimental approaches to stimulate the ECS as a therapeutic target in toxic paradigms with excitotoxic components.

Oleamide (*Cis*-9,10-Octadecenoamide, or ODA) is a fatty acid amide and an endocannabinoid-profiled compound which is abundant in the CNS (Fowler 2004). First described as an endogenous sleep-inducing substance (Cravatt et al. 1995), ODA was further characterized as a molecule that modified different physiological responses controlled by the CNS, including analgesia, memory (Murillo-Rodríguez et al. 2001; Akanmu et al. 2007), and locomotion (Huitrón-Resendiz et al. 2001). At the CNS level, ODA is known to inhibit gap junction (connexin)-mediated cell-cell communication (Boger et al. 1998) and modulates serotonergic 5-HT_1, $5HT_{2A/2C}$, and 5-HT_7 receptors (Mueller and Driscoll 2009), as well as γ-amino butyric acid ($GABA_A$) receptors (Verdon et al. 2000). In addition, this molecule has been shown to be responsible for some neuroprotective effects, such as the reduction of amyloid-β (Aβ) peptide accumulation and the subsequent inflammatory response in a murine model of Alzheimer's disease via calpain inhibition (Ano et al. 2015). More recently, the antiepileptic and neuroprotective effects of ODA in the striatum were shown in rats exposed to kainic acid in an excitotoxic model (Nam et al. 2017). Altogether, these effects suggest a wide neuroprotective potential of ODA under different experimental conditions and a possible protective role in excitotoxic models. However, the amount of evidence on this topic remains scarce. Moreover, while some reports support the concept that ODA can act as an agonist on the CB1 receptor, as it is structurally similar to the endocannabinoid anandamide (Boger et al. 2000; Leggett et al. 2004), others have suggested that this amide exerts no effects on the CB1 receptor (Mechoulam et al. 1997; Lichtman et al. 2002). Therefore, in this work, we evaluated whether ODA is able to exert neuroprotective effects in the toxic model produced by QUIN in rat brain synaptosomes and cortical slices, and if these effects are subordinated to CB1 activation. In addition, a possible role of CB2 receptors was explored as part of the protective pattern exerted by ODA. For these purposes, we obtained fresh crude synaptosomal/mitochondrial fractions and cortical slices and incubated both biological preparations in the presence of QUIN to produce excitotoxicity. We also challenged this model with ODA ± AM281 (a selective CB1 receptor antagonist/inverse agonist) or JTE-907 (a selective CB2 inverse agonist), in order to explore a possible role of cannabinoid receptors in the protective effects of the amide. Our results suggest that the protective effects exerted by ODA in the excitotoxic events elicited by QUIN are partially mediated by activation of CB1 receptors, and in a more extent, by CB2 receptors.

Materials and Methods

Reagents

All reagents were of analytical grade. ODA, QUIN, HEPES, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), thiobarbituric acid (TBA), and other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO). 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) were obtained from Thermo Fisher Scientific (Waltham, MA). AM281 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1H-pyrazole-3-carboxamide) and JTE-907 (*N*-(1,3-benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2-oxo-8-(pentyloxy)-3-quinolinecarboxamide) were purchased from Tocris Bioscience (Bristol, UK). Salts for buffer preparation were obtained from JT Baker (Avantor Performance Materials, Center Valley, PA, USA). Other chemicals were obtained from other commercial sources. All solutions were prepared in deionized water obtained from a Milli-RQ system (Millipore, MA).

Animals

Adult male (250–300 g; $N = 45$) Wistar rats were obtained from local breeding stocks. Animals were housed five per cage in acrylic cages and provided with food and water ad libitum in the vivarium of the Instituto Nacional de Neurología y Neurocirugía (National Institute of Neurology and Neurosurgery, Mexico). Temperature (25 ± 3 °C), humidity, and light cycles (12:12 light/dark schedule) were maintained constant over the housing period. All experimental manipulations of the animals were performed in accordance with the "Guidelines for the Use of Animals in Neuroscience Research" from the Society of Neuroscience, and in compliance of the ARRIVE guidelines. The experimental protocols were approved by the Ethics Committee for Animal Research of the Instituto Nacional de Neurología y Neurocirugía (Project No. 126/17). All efforts were made to minimize the number of animals used and their suffering during the experiments.

Preparation and Treatment of Crude Synaptosomal Fractions

Isolated synaptosomes were considered as crude synaptosomal/mitochondrial P2 fractions. These fractions were obtained from the brains of adult rats, according to a method reported previously by us (Rangel-López et al. 2015). Once obtained after serial centrifugations, synaptosomes were incubated in a shaking water bath at 37 °C in the presence of 100 μM QUIN for 60 min. Additional synaptosomal fractions were preconditioned for 30 min with increasing concentrations of ODA (1–50 μM) and/or 1 μM AM281 (added 15 min before ODA), a selective CB1 antagonist/inverse agonist. While the toxic concentration of QUIN for the assays with synaptosomes and slices was chosen from a previous report (Rangel-López et al. 2015), the ODA concentration range tested in both preparations was inferred from other bibliographic sources using this agent under different in vitro conditions (Gobbi et al. 1999; Verdon et al. 2000; Dougalis et al. 2004; Lee et al. 2006). The AM281 concentration was also inferred from previous reports using this agent under in vitro conditions (Lan et al., 1999; Li et al., 2010; García-Morales et al., 2014; Sánchez-Rodríguez et al., 2018). After incubation, all samples were prepared for the estimation of toxic endpoints. The protein content in all samples was quantified by Lowry's method (Lowry et al. 1951).

MTT Reduction Assay as a Functional Assessment in Synaptosomes

MTT reduction was assessed in the mitochondrial-enriched synaptosomal fractions, according to a previous report (Colín-González et al. 2015). The formazan content in samples was quantified in a CYT3MV Biotek Cytation 3 Imaging Reader at a 570 nm wavelength. Results were expressed as percent of total MTT reduction vs. control values.

Lipid Peroxidation Assay as a Functional Index of Oxidative Damage in Synaptosomes

Lipid peroxidation was quantified as an index of oxidative damage to lipids in synaptosomal/mitochondrial fractions by TBA-reactive substance (TBARS) detection, according to a previous report (Colín-González et al., 2015). After exposure to QUIN (100 μM) and/or ODA (1 μM), synaptosomal fractions (250 μl-aliquots) were incubated for 30 min at 94 °C in the presence of 200 μL of the TBA reagent (0.75 g of TBA + 15 g of trichloroacetic acid + 2.54 mL of HCl) and further ice-cooled for 5 min. Optical density in samples was measured at 532 nm in a CYT3MV Biotek Cytation 3 Imaging Reader. Results were calculated as nmol of MDA (equivalents) per mg protein, normalized, and expressed as percent of lipid peroxidation compared with the control.

Isolation and Treatment of Brain Cortical Slices

Cortical slices were obtained according to a method previously described (Colín-González et al. 2014). After rats were euthanized by decapitation and their brains dissected out, the whole frontal cortex was maintained in a Krebs buffer containing 124 mM NaCl, 5 mM KCl, 1.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 23 mM NaHCO3, 3 mM HEPES, and 10 mM D-glucose with 95% O_2/5% CO_2 (pH 7.4). Four slices were collected per probe (250–300 μm thickness). Protein quantification was carried out according to the method described by Lowry et al. (1951). Cortical slices received ODA (10–100 μM) as preconditioning for 60 min, and then, QUIN (100 μM) was added to the slices and incubated for 60 more min, all this, in a shaking water bath at 37 °C. Additional slices exposed to ODA + QUIN also received the selective CB1 antagonist/inverse agonist AM281 (1 μM; added 15 min before ODA) as preconditioning to the toxic insult. An additional set of experiments was carried out aimed at testing a possible role for CB2 receptors in the effect of ODA. For this purpose, ODA + QUIN-treated slices received the selective CB2 inverse agonist JTE-907 (0.38 nM; added 15 min before ODA) also as preconditioning to the toxic insult. The JTE-907 concentration used in these experiments was inferred from a previous study (Iwamura et al. 2001).

Functional Assessment of Cell Viability in Cortical Slices

The MTT reduction assay was assessed according to a method previously described (Colín-González et al. 2014). After

being incubated with all treatments, the slices were re-incubated at 37 °C for 60 min in the presence of MTT (5 mg/ml) and the samples were then centrifuged at 15,300×*g* for 15 min. The corresponding pellets were collected and resuspended in 1 ml of isopropanol. After a second centrifugation step (1700×*g* for 3 min), the content of formazan was measured in the supernatants in a CYT3MV Biotek Cytation 3 Imaging Reader at a 570 nm wavelength. Results were expressed as the percent of MTT reduction vs. the control values.

Cell Damage Assessment in Cortical Slices

To differentiate between "healthy" cells and cells undergoing damage, a simple method based on immunofluorescence was used, according to a previous report (Ortega et al. 2015). Briefly, cortical slices previously exposed to different experimental conditions were directly loaded with PI (100 μg/ml, Roche) for 10 min; the medium was removed at this point, and slices were PBS-washed three times, fixed with paraformaldehyde (Sigma, 1%/PBS) for 60 min and mounted using a DAPI-resin for nuclei counter-stain. The slices were observed using Cytation 3 equipment (Bioteck), and images were analyzed by the software provided by the supplier (Gen5 v3.02.2). Results were presented as images of simple and double staining (merge) for DAPI and PI.

Statistical Analysis

Results are expressed as mean values ± S.E.M. Bars in all figures represent the average of six assays (except for Fig. 4—three assays) per experiment (one rat per assay, all treatments run in parallel in each experiment). Data were statistically analyzed by two-way analysis of variance (ANOVA), followed by Bonferroni's test. Values of $P \leq 0.05$ were considered statistically significant. All analytical procedures were calculated using the GraphPad Prism 5 software (GraphPad Scientific, San Diego, CA, USA).

Results

ODA Prevented the QUIN-Induced Loss of Mitochondrial Reductive Capacity in Rat Brain Synaptosomes in a Partial CB1 Receptor Manner

In this first experimental approach, we investigated whether ODA is capable of preventing the QUIN-induced loss of mitochondrial function in brain synaptosomes (Fig. 1). In the upper panel, mitochondrial activity remained unchanged in synaptosomes incubated in the presence of increasing concentrations (1–50 μM) of ODA alone. In the middle panel, QUIN decreased the mitochondrial activity by 45% below the

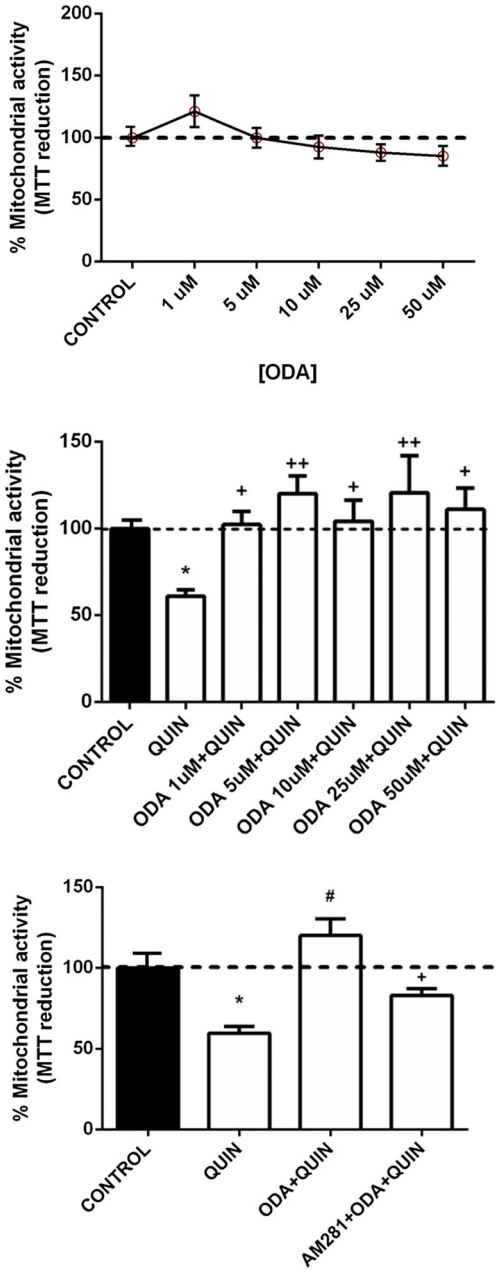

Fig. 1 Effect of oleamide (ODA) on the quinolinic acid (QUIN)-induced loss of mitochondrial reductive capacity in rat brain synaptosomal/mitochondrial fractions. Mitochondrial activity was assessed by the MTT reduction assay. Dots and bars in the graphs depict the percent of MTT reduction in synaptosomes preconditioned for 30 min with ODA (1–50 μM) and/or AM281 (1 μM; added 15 min before ODA), and further exposed for 60 min to QUIN (100 μM). In the upper panel, the effect of increasing concentrations of ODA on MTT reduction is compared vs. the control. In the middle panel, the effect of increasing concentrations of ODA on the QUIN-induced depleted MTT reduction is shown. In the bottom panel, the effect of AM281 on the ODA (5 μM) + QUIN-induced decreased MTT reduction is shown. Mean values ± S.E.M. of six assays per group (3 repeats per assay). $^{*}P \leq 0.05$, different from the control group in all panels; $^{+}P \leq 0.05$ and $^{++}P \leq 0.01$, different from QUIN in the right upper panel; $^{\#}P \leq 0.01$, different from QUIN in the bottom panel; $^{+}P \leq 0.05$, different from ODA + QUIN in the bottom panel; two-way ANOVA followed by Bonferroni's test

control ($P \leq 0.05$, different from the control condition), whereas ODA completely prevented this effect, keeping mitochondrial function similar (3%, 5%, and 8% above the control for 1, 10, and 50 μM, respectively; $P \leq 0.05$, different of QUIN) or even above the control (18% and 19% above for 5 and 25 μM, respectively; $P \leq 0.01$, different from QUIN). In the bottom panel, QUIN decreased mitochondrial function again by 44% below the control ($P \leq 0.05$, different from the control group), whereas ODA (5 μM) prevented this effect by increasing mitochondrial function by 20% above the control ($P \leq 0.01$, different of QUIN). The CB1 receptor antagonist/inverse agonist AM281 partially recovered the QUIN toxicity by decreasing mitochondrial function compared with the ODA + QUIN treatment (30% below; $P \leq 0.05$, different of the ODA + QUIN treatment), though this effect was close to the control value (N.S., compared with the control; $P \leq 0.05$, different from QUIN).

ODA Reduced the QUIN-Induced Lipid Peroxidation in Rat Brain Synaptosomes

In order to know whether the protective spectrum of ODA on QUIN-exposed synaptosomes include the reduction of oxidative stress, a simple test sensing the levels of oxidative damage to lipids was carried out. In Fig. 2, QUIN increased lipid peroxidation by 61% above the control ($P \leq 0.05$, different from control), whereas pretreatment of synaptosomes with ODA (1 μM) decreased the QUIN-induced oxidative damage

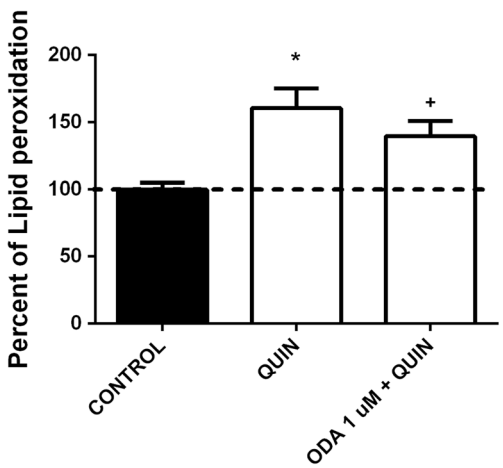

Fig. 2 Effect of oleamide (ODA, 1 μM) on the quinolinic acid (QUIN)-induced oxidative damage to lipids in rat brain synaptosomal/mitochondrial fractions. Lipid peroxidation was assessed by the thiobarbituric acid (TBA)-reactive substance assay. Bars in the graph depict the levels of lipid peroxidation (expressed as the percent of oxidative damage to lipids) at 60 min after being exposed to QUIN (100 μM). Mean values ± S.E.M. of six assays per group (three repeats per assay). $^{*}P \leq 0.05$, different from the control group; $^{+}P \leq 0.05$, different from QUIN; two-way ANOVA followed by Bonferroni's test

by 14%, compared with QUIN ($P < 0.05$, different from QUIN). ODA alone did not modify the basal levels of lipid peroxidation (data not shown).

ODA Attenuated the QUIN-Induced Decrease of Cell Viability in Rat Cortical Slices in a Partial CB1 Receptor Manner

The effect of ODA on the QUIN-induced decrease in cell viability was investigated in cortical slices (Fig. 3). In the left panel, QUIN (100 μM) decreased cell viability by 74% below the control ($P \leq 0.0001$), whereas incubation of the slices with ODA (10, 25, 50 75, and 100 μM) administered as a pretreatment before QUIN, resulted in significant prevention of the effect of the toxin (25%, 19%, 40%, 22%, and 4% below the control, respectively; $P \leq 0.0001$, different from QUIN). In fact, 100 μM ODA totally reversed the effect of QUIN. The right panel shows the protective effect induced by 25 μM ODA on the QUIN-induced toxicity, which was partially prevented by the addition of AM281 (41% below the control; $P \leq 0.0001$, different from the control; $P \leq 0.0001$, different from ODA + QUIN), thus suggesting a partial involvement of CB1 receptor in the process. Neither ODA (7% above the control) nor AM281 (data not shown) modified cell viability in the slices compared with the control condition.

ODA Prevented the Cell Damage Induced by QUIN in a Partial CB1 Receptor Manner

Fluorescence micrographs showing the effect of ODA on the QUIN-induced cell damage are shown in Fig. 4. The control condition (first line) shows strong DAPI staining contrasting with moderate PI staining, meaning that cell permeability is not compromised in this condition, as evidenced by the merge image. In contrast to the control condition, slices exposed to QUIN (second line) showed strong PI staining merging with DAPI labeling (PI/DAPI ratio = 653% above the control; $P \leq 0.001$, different from the control). In the third line, ODA alone (25 μM) did not induce any significant PI staining. The incubation of the slices in the presence of ODA + QUIN (fourth line) produced a moderate increase in the PI staining compared with the control (PI/DAPI ratio = 307% above the control; $P \leq 0.05$, different from the control), suggesting a decrease in cell permeability compared with the QUIN condition (PI/DAPI ratio = 56% below QUIN; $P \leq 0.01$, different from QUIN). Preconditioning of the ODA + QUIN-treated slices with AM281 resulted in increased PI staining compared with the ODA + QUIN condition (PI/DAPI ratio = 51% above the ODA + QUIN condition; $P \leq 0.001$, different from the control), supporting a partial prevention of the protective effects of ODA. A positive control of cell damage was run in parallel and consisted in the incubation of the slices in the presence of H_2O_2, which caused strong PI staining (742%

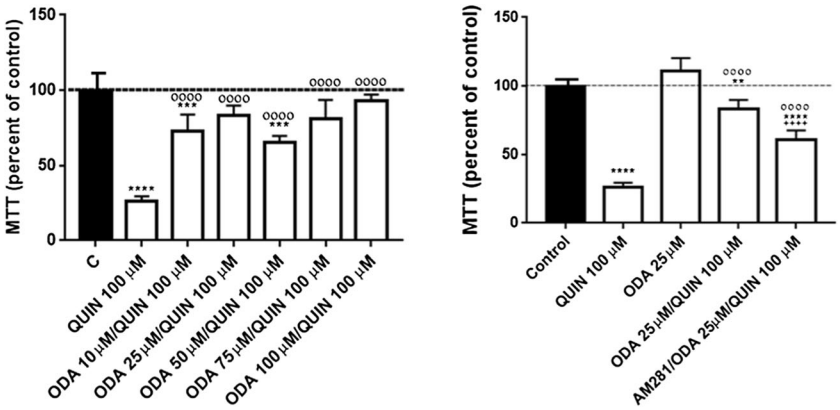

Fig. 3 Effect of oleamide (ODA) on the quinolinic acid (QUIN)-induced loss of cell viability in rat cortical slices. Cell viability was assessed by the MTT reduction assay. Bars in the graph depict the percent of MTT reduction in slices preconditioned for 60 min with ODA (10–100 μM) and/or AM281 (1 μM; added 15 min before ODA), and further exposed for 60 min to QUIN (100 μM). In the left panel, the effect of increasing ODA concentrations (10–100 μM) on QUIN toxicity is shown. In the right panel, the effects of ODA (25 μM) and/or AM281 (1 μM) on QUIN toxicity are depicted. Mean values ± S.E.M. of six experiments per assay (three repeats per assay). $^{**}P \leq 0.01$ and $^{****}P \leq 0.0001$, different from the control group in all panels; $^{\circ\circ\circ\circ}P \leq 0.0001$, different from QUIN in all panels; $^{++++}P \leq 0.0001$, different from ODA + QUIN in the right panel; two-way ANOVA followed by Bonferroni's test

above the control; $P \leq 0.001$, different from the control). AM281 alone did not induce any change in PI staining, compared with the control group (data not shown). The densitometric analysis of these images, supporting the above-described observations, is represented in the graph at the bottom of Fig. 4.

The Protective Effect of ODA on the QUIN-Induced Decrease of Cell Viability in Rat Cortical Slices Is Also Mediated by CB2 Receptor Activation

Once the protective effect of ODA was partially linked with CB1 receptor activation (from our previous experiments), we further investigated whether CB2 receptors could also be involved in the actions of ODA through a simple experiment, challenging ODA + QUIN-treated cortical slices with the CB2 inverse agonist JTE-907 (Fig. 5). QUIN (100 μM) decreased cell viability by 76% below the control ($P \leq 0.0001$, different from the control), while ODA (25 μM) prevented again the QUIN-induced loss of cell viability (24% below the control; $P \leq 0.0001$, different from the control). Pre-incubation of ODA + QUIN-treated slices with JTE-907 significantly reduced the protective effect of ODA (55% below the control; $P \leq 0.0001$, different from the control; $P \leq 0.001$, different from ODA + QUIN), thereby supporting partial involvement of CB2 in the process. It is noteworthy that the effect of JTE-907 was more prominent than that produced by the CB1 inverse agonist AM281 (Fig. 3). In addition, searching a possible additive or synergistic action of JTE-907 plus AM281, ODA + QUIN-treated slices were exposed to these two agents. The effect of this combined condition did not differ from the JTE-907 + ODA-QUIN treatment (53% below the control; $P \leq 0.0001$, different from the control; $P \leq 0.001$, different from ODA + QUIN). JTE-907, per se, did not alter the baseline levels of cell viability observed in the control condition.

Discussion

Novel therapeutic approaches are needed for limiting the progression of degenerative events in neurological disorders. The pharmacological manipulation of ECS is a promising target for the design of therapeutic approaches in human disorders (Robson 2014). It has been suggested that the protective effects produced by pharmacological manipulation of the ECS are linked to the stimulation of CB1, CB2, and/or other receptors. In this regard, it is known that CB1 modulates the activity of NMDAr by a mechanism that involves the reduction of NR1 subunits by the CB1-coupled protein HINT1 (Sánchez-Blázquez et al. 2014), thereby limiting the effects of the excitatory glutamatergic activity, as suggested in previous studies (Rangel-López et al. 2015; Kotlar et al. 2019; Aguilera-Portillo et al. 2019). Together with the presynaptic inhibition of neurotransmitter release, this novel mechanism supports a role for the CB1 receptor in glutamatergic terminals as a major target to reduce and control excitotoxic damage produced by excessive glutamatergic activity (Chiarlone et al. 2014). Under this mechanistic scenario, for the first time, we describe here the neuroprotective properties of ODA in a typical excitotoxic acute model produced by the glutamate analogue QUIN in two biological preparations involving neuronal activity at two different functional levels: synaptic transmission in synaptosomal fractions, and the whole complexity of a brain slice encompassing not only neurons, but all other cell types. It is noteworthy that ODA exerted protection against the loss of mitochondrial and cellular reductive capacity in both preparations, also decreasing the cell damage in the

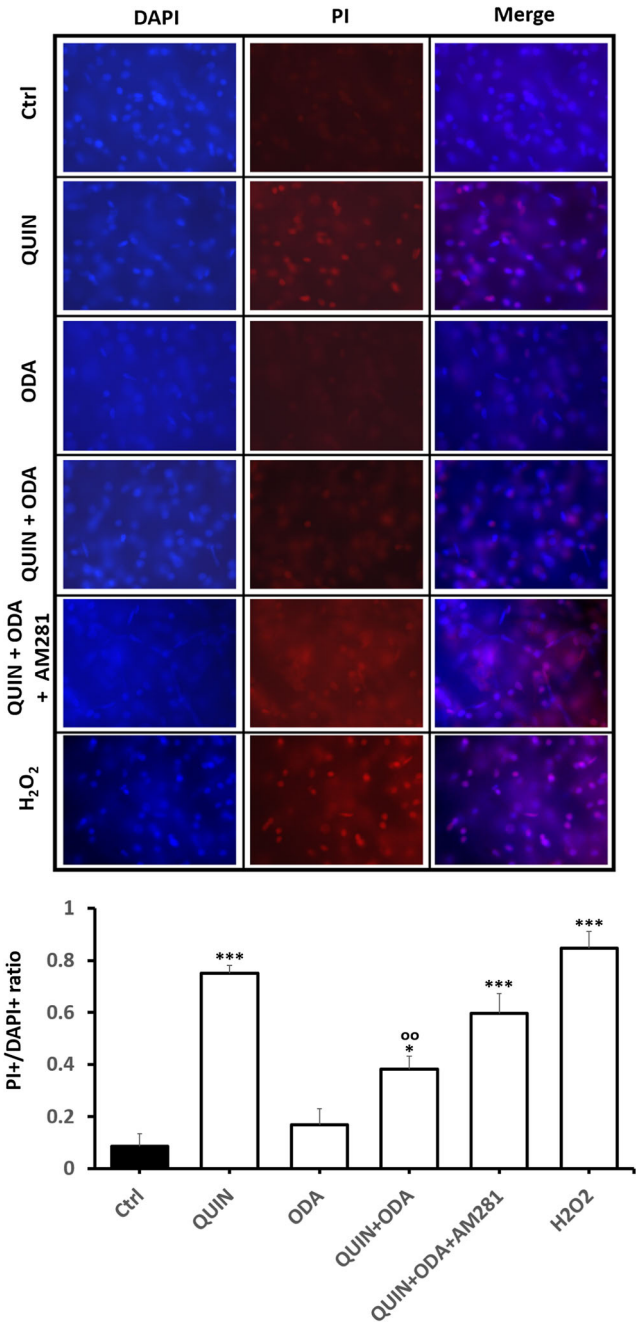

Fig. 4 Effect of oleamide (ODA) on the quinolinic acid (QUIN)-induced cell damage in rat cortical slices. In the first column, fluorescence micrographs show cell nuclei of slices exposed to H_2O_2 (positive control), QUIN (100 μM), ODA (25 μM), and/or AM281 (1 μM), stained with 4′,6-diamidino-2-phenylindole (DAPI). In the second column, propidium iodide (PI) fluorescence staining of cells subjected to the same treatments is depicted. The third column shows the DAPI and PI merge. Bar lines correspond to 100 μm. All fields correspond to × 20 magnifications. The graphic representation of the densitometric analysis of the images from the upper panels is shown below. Mean values ± S.E.M. of three experiments per assay. $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$, different from the control group; $^{oo}P \leq 0.01$, different from QUIN; two-way ANOVA followed by Bonferroni's test

slices, thus suggesting a broad spectrum of protective actions at different cell levels. In fact, similar effects have been

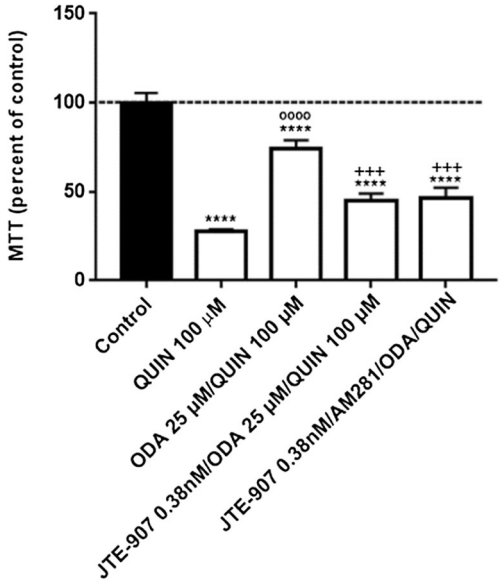

Fig. 5 Effect of JTE-907 on the oleamide (ODA)-mediated prevention of decreased cell viability induced by quinolinic acid (QUIN) in rat cortical slices. Cell viability was assessed by the MTT reduction assay. Bars in the graph depict the percent of MTT reduction in slices preconditioned for 60 min with ODA (10–100 μM) ± JTE-907 (0.38 nM; added 15 min before ODA) and/or AM281 (1 μM; added 15 min before ODA), and further exposed for 60 min to QUIN (100 μM). Mean values ± S.E.M. of six experiments per assay (three repeats per assay). $^{****}P \leq 0.0001$, different from the control group; $^{oooo}P \leq 0.0001$, different from QUIN; $^{+++}P \leq 0.001$, different from ODA + QUIN; two-way ANOVA followed by Bonferroni's test

observed in a recent report testing another endocannabinoid, anandamide, on the toxic actions elicited by QUIN, glutaric acid (GA), and QUIN + GA in cultured neuronal cells (Kotlar et al. 2019), further highlighting the beneficial role that stimulation of the ECS exerts against neurotoxic paradigms involving excitotoxicity. Nonetheless, the lack of a clear concentration-response effect of ODA in our experiments—ODA induced high protection even with the lowest concentration tested—clearly suggests that lower concentrations should be tested in further studies. Unfortunately, since no previous reports exist on this specific issue with ODA, the rank of concentrations tested here was inferred merely on the basis of other studies in our group testing other cannabinoids (Kotlar et al. 2019). Furthermore, derived from our results, the protective effect of ODA seems to involve, at least in part, activation of CB1 receptors, as evidenced by the partial effect exerted by the CB1 antagonist/inverse agonist AM281 on ODA + QUIN-exposed synaptosomal fractions and cortical slices. In this regard, it is pertinent to mention that this study was originally designed to test the potential involvement of CB1 receptor activation on the protective pattern evoked by ODA; however, given the partial contribution of the CB1 receptor, we also decided to explore the role of CB2 receptors on the effects of ODA. It is noteworthy that the blockade of CB2 receptors by the inverse agonist JTE-907 resulted in a stronger

inhibition of the protective action of ODA, compared with CB1 receptor blockade, albeit the partial effect of the inverse agonist supports the notion that not all the positive actions of ODA can be explained by CB2 receptors, nor by CB1 receptors. In this context, CB2 receptors might induce such a degree of protection by modulating and reducing the aberrant signals linked with intracellular Ca^{2+} in the toxic model, and this effect is suggested by the ability of CB2 receptors to form heterodimers with the G protein-coupled chemokine receptor, CXCR4, a protein widely expressed in the brain (van der Meer et al. 2000) in charge of intracellular Ca^{2+} mobilization (Coke et al. 2016). In turn, this and other mechanisms could attenuate the QUIN toxicity by reducing postsynaptic synaptic Ca^{2+} signaling. Moreover, it is likely that CB1 and CB2 receptors are not acting in a concerted manner to induce protective responses when activated by ODA in toxic insults, judging by the lack of additive or synergistic effects when both selective inverse agonists were simultaneously added to ODA + QUIN-treated slices. Thus, considering the partial and non-additive effects evoked in this study by selective CB1 and CB2 inverse agonists, AM-281 and JTE-907, respectively, we hypothesize that other receptors and/or mechanisms might be involved in the effects produced by ODA, including vanilloid receptors, 5HT2c receptors, among several others. These alternatives cannot be discarded at all and deserve detailed characterization.

Of further interest is the fact that, as a clear effect of excitotoxic damage, cells can compromise their functions, starting with membrane permeability, even under a short-term toxic exposure. This compromise will further lead to cell death and neurodegeneration (Mattson et al. 1999). Consistent with this concept, in this study, the exposure of cortical slices to QUIN displayed disrupted permeability and enhanced cell damage, demonstrating that cells submitted to excitotoxic insults are physiologically compromised since the beginning of the exposure. Since ODA was able to reduce this effect, it seems feasible to suggest that the protective properties of this amide comprise the preservation of key cell functions, such as membrane permeability, even with short-term toxicity.

To our knowledge, besides the present report, there are only a few articles in literature dealing with the concept that ODA can be neuroprotective. Antiepileptic and neuroprotective effects of ODA were demonstrated in the striatum of rats subjected to kainic acid-induced excitotoxic brain damage (Nam et al. 2017). Rats given oral administration of ODA exhibited inhibitory dose-dependent effects on KA-induced seizures, morphological alterations, and calpain activation. In the same report, calpain inhibition was demonstrated in in vitro experiments as a direct effect of ODA. Considering these results, it is likely that the inhibition of calpain by ODA accounts for the observed neuroprotective actions, albeit this effect was also partially dependent on CB receptors. Therefore, it is feasible to consider the protective mechanism described by Nam et al. (2017) as an additional and not excluding event mediated by ODA. Moreover, ODA has been shown to block Gap junctions under pathological conditions, thereby reducing the propagation of apoptosis (Mueller and Driscoll 2009). Furthermore, according to Oh et al. (2010), the effects of ODA are likely to recruit the regulation of several signaling pathways to evoke anti-inflammatory responses. The effects of ODA on the lipopolysaccharide (LPS)-induced production of inflammatory mediators—including NF-κB activation, nitric oxide and prostaglandin E2 accumulation, and iNOS and COX-2 expression—in activated microglia via inhibition of Akt, p38 MAPK, and ERK phosphorylation, as well as PI 3-kinase activation, all in a CB2 receptor-dependent manner, were tested by these authors. More recently, the capacity of ODA to suppress inflammatory responses in microglial and human dendritic cells via CB2 receptor activation was demonstrated (Kita et al. 2019). These findings are particularly relevant for our report to explain why the protective effects of ODA in the cortical slices are so prominent, as this biological preparation, in contrast to synaptosomes, recruits different cell types, including neurons, microglia, astrocytes, and endothelial cells, among others. Thus, while the results of this study strengthen the notion that ODA can protect the brain, in part through CB1-CB2-mediated mechanisms, its effect in slices and synaptosomal fractions might also recruit the activation of other mediators such as vanilloid, PPAR-γ, and/or other receptors. The neuroprotective properties elicited by the activation of CB1 and CB2 receptors are of considerable value for toxicological research, especially when considering that CB1 receptors are widely located in neurons, while CB2 receptors are present both in glial cells (Nazari et al. 2016), and in nerve endings, also in a functional manner (Pascual et al. 2014). These strategic localizations are critical for the occurrence of modulatory signals and regulatory mechanisms, ultimately accounting for the preservation of neuronal homeostasis.

In regard to the moderate (yet significant) preventive effect exerted by ODA on the QUIN-induced oxidative damage to lipids in synaptosomes, we hypothesize that, since antioxidant activity is not the primary property of this amide, the reduction of lipid peroxidation observed here is probably due to indirect events, such as the prevention of massive Ca^{2+} influx and the subsequent early blockade of excitotoxicity.

In summary, altogether, the experimental evidence reported here makes it plausible to hypothesize that, besides its well-known sleep-inducing properties, ODA might be considered as a potential neuroprotective fatty acid metabolite, though more detailed experimental evidence is needed to support this notion. Furthermore, based on the results obtained in this study, and the increasing amount of scientific literature on this topic, the design and use CB-based therapies for the treatment of human disorders is gaining attention. In addition, the results of the present study support the notion that the pharmacological manipulation of the ECS constitutes a key target to design specific therapeutic approaches for the treatment of

debilitating neurological disorders that depend, at least in part, on excitotoxic events.

Concluding Remarks

Here, for the first time, we provide experimental evidence supporting a neuroprotective role of the neuromodulatory cannabinoid-profiled molecule, ODA, on short-term toxicity induced by the excitotoxic metabolite, QUIN, in rat brain synaptosomal fractions and cortical slices. This protective effect involves, at least in part, the activation of CB1 and CB2 receptors. These facts highlight the relevance of the ECS for the preservation of the homeostatic status in the brain when confronting possible toxic insults. The spectrum of toxic events that were efficiently prevented by ODA comprise synaptic and cell dysfunction at the mitochondrial level, oxidative damage to lipids, and general cell damage. Thus, the protective effects exerted by ODA in the toxic model produced by QUIN prompt detailed investigation on the therapeutic potential that cannabinoid-based approaches offer for some neurodegenerative disorders.

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Compliance with Ethical Standards

The experimental protocols were approved by the Ethics Committee for Animal Research of the Instituto Nacional de Neurología y Neurocirugía (Project No. 126/17).

Conflict of Interest The authors declare that they have no competing interests.

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