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CRIP1a switches cannabinoid receptor agonist/antagonist-mediated protection from glutamate excitotoxicity☆

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ABSTRACT

A shared pathology among many neurological and neurodegenerative disorders is neuronal loss. Cannabinoids have been shown to be neuroprotective in multiple systems. However, both agonists and antagonists of the CB_1 cannabinoid receptor are neuroprotective, but the mechanisms responsible for these actions remain unclear. Recently a CB_1 receptor interacting protein, CRIP1a, was identified and found to alter CB_1 activity. Here we show that in an assay of glutamate neurotoxicity in primary neuronal cortical cultures CRIP1a disrupts agonist-induced neuroprotection and confers antagonist-induced neuroprotection.

The CB_1 cannabinoid receptor is the most abundant G protein coupled receptor (GPCR) in the brain [15] and accordingly it has roles in many neural functions, including memory, analgesia, appetite, addiction, synaptic plasticity and protection against neuronal cell death during seizures [16,19,21,22]. Protection against seizure-induced cell death in mice is greater with higher CB_1 expression and depends on CB_1 cannabinoid receptors located specifically in glutamatergic forebrain neurons [12,22]. Activation of CB_1 receptors also protects hippocampal, striatal, and motor neurons from excitotoxicity. Cell death after excitotoxic treatments including reduced Mg^{2+}, kainic acid, glutamate, or NMDA and in Huntington's disease or cerebral ischemia was reduced by CB_1 receptor expression and activity [1,4,13,17,24,26,29,31,33].

Although CB_1 cannabinoid receptor activation protected neurons against toxic insults, including those resulting from excitotoxicity [1,26,29], ischemia [24,26,31], and seizure [22], activation also induced cell death [9,27]. Further, inhibition of the CB_1 cannabinoid receptor protected against ischemia [2,23,32] and excitotoxicity [14]. The mechanisms for these paradoxical results remain poorly defined. The complex roles of the CB_1 receptor, its involvement in numerous neuronal systems, and experimental differences add to the ambiguity of CB_1 receptor's role in neuroprotection.

The mechanisms underlying CB_1 receptor-mediated neuroprotection are unclear but are potential therapeutic targets in the treatment of cell death that occurs after excitotoxic insults including those in chronic epilepsy, Huntington's disease, and ischemia. GPCRs interact with and are regulated by numerous interacting proteins that play important roles in elemental receptor functions including receptor trafficking, signal transduction, and desensitization. We recently identified a cannabinoid receptor interacting protein, CRIP1a [25]. CRIP1a was found to bind CB_1, but not CB_2 cannabinoid receptors. CRIP1a did not alter CB_1 expression, ligand binding affinity, protein maturation or membrane localization. Instead, CRIP1a was found to reduce the CB_1-mediated tonic inhibition of voltage-gated calcium currents [25]. In the present study we tested whether CRIP1a can modulate neuroprotection by cannabinoid agonists and antagonists in an *in vitro* model of glutamate neurotoxicity. We report that CRIP1a switches the neuroprotective effect of a cannabinoid agonist to neuroprotection by a cannabinoid antagonist.

Cortical cultures: Embryonic day 18 (E18) cortical cultures from Long Evans rats were established as in [28]. Cells were plated at 1.86×10^5 cells/cm^2 onto poly-D-lysine and laminin-coated 24-well plates and maintained at 37 °C in Basal Media Eagle, 1 mM glutamine, 1%N2, and 5%FBS. Media was replaced every 3–4 days.

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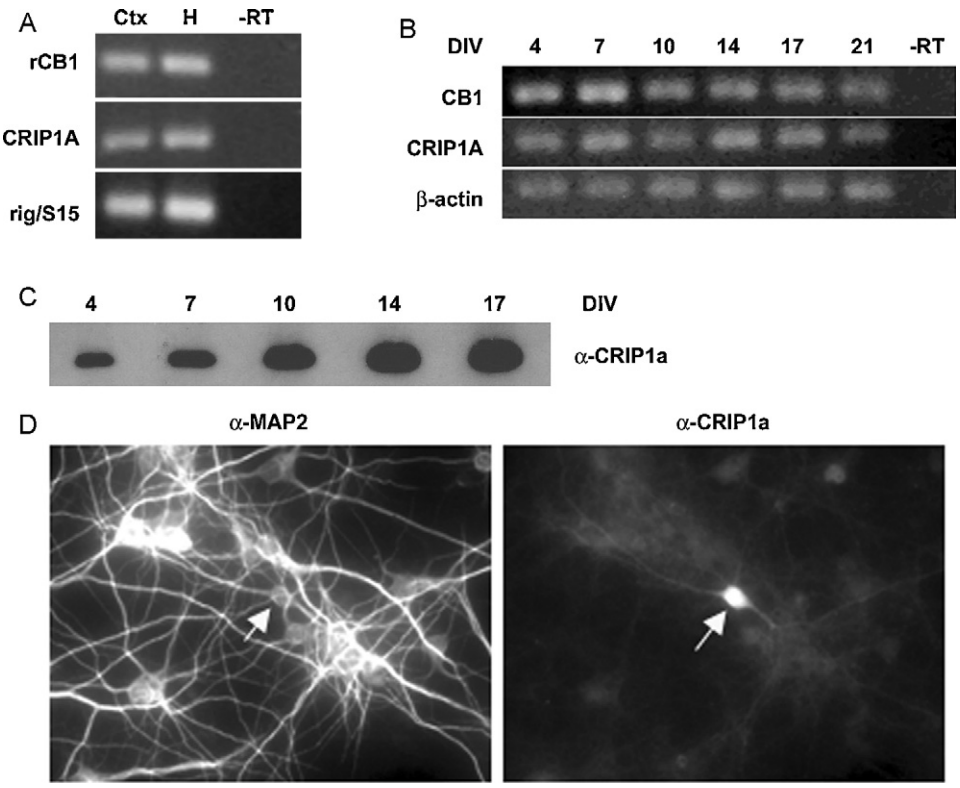

Fig. 1. CB_1 and CRIP1a are expressed in cortical neurons *in vitro* and *in vivo*. (A) RT-PCR analyses of rat CB_1, CRIP1a, and rig/S15 in RNA isolated from E18 cortex (C) and hippocampus (H). PCR on RNA that was not reverse transcribed (-RT). (B) RT-PCR analyses for expression of rat CB_1, CRIP1a, and β-actin in RNA isolated from E18 rat cortical cultures at the DIV indicated. (C) Western blot analysis with CRIP1a specific antibodies (α-CRIP1a) of E18 cortical cultures harvested at the DIV indicated. (D) Images of E18 cortical neurons at 14DIV immunostained with MAP2 (α-MAP2) and CRIP1a (α-CRIP1a) specific antibodies. Arrows indicate a co-immunostained neuron.

RT-PCR: Total RNA was isolated from E18 tissue using FastRNA Pro Green Kit (Q-BIOgene) and from cultured cells using Ultraspec RNA Isolation Reagent (Biotecx) following manufacturer's instructions. To remove genomic DNA, 2 μg of total RNA was incubated with RNase free DNase for 30 min at 26 °C. EDTA was added to final concentration of 1 mM, the mixture was heated at 65 °C for 15 min then cooled on ice. Reverse transcription was performed in 40 μl by adding to the RNA sample 2 μl RNAse inhibitor, 2 μl random primers, 8 μl 10 mM dNTPs, 4 μl reverse transcription buffer, and 2 μl MMLV reverse transcriptase. The reaction mixture was incubated at 42 °C for 1 h, 92 °C for 10 min then incubated with 1.5 μl RnaseH at 37 °C for 20 min, centrifuged, and stored at −20 °C. PCR was performed using 1 μl of the cDNA for 30 cycles with CB_1 and CRIP1a specific primers. Rig/S15 (rat insulinoma gene/ribosomal protein S15) and β-actin 'housekeeping' genes were amplified as endogenous controls. RT-PCR was performed two independent times from two independent samples. To control for genomic contamination, reverse transcriptase was excluded in parallel PCR reactions.

Immunocytochemistry: Cultures were fixed and blocked as in [28], immunostained for CRIP1a (rabbit antiserum 077.4 diluted 1:1000, generated as in [25]) and MAP2 (1:2000; Sigma), and visualized by Alexa-488- and Alexa-568-conjugated secondary antibodies (Invitrogen). Images were acquired from 2 to 4 independent experiments using an Axiovert200 Zeiss fluorescent microscope with 40× objective.

Western blot: Cells were homogenized in 150 mM NaCl, 1%SDS, 0.02%NaN3, 1%NP-40, 0.5%DOC and 50 mM Tris pH 8.0 containing 100 μg/ml PMSF and one protease inhibitor pellet (Roche). BCA assays determined protein concentrations. 3 μg of each sample was loaded onto SDS/PAGE gels, transferred to PVDF membranes, probed with CRIP1a antiserum (1:4000) in 1%milk, 1%BSA, 0.05%Tween-20 in TBS, secondary antibody, and visualized by ECL.

Lentiviral construction and infection of cortical cultures: A self-inactivating lentivirus transfer vector and three plasmids expressing structural and regulatory elements were generously supplied by N. Déglon of Lausanne University [6,7]. A control transfer vector (SSWG-EGFP) and one encoding rat CRIP1a (SSWG-CRIP1a) under control of the neuron-specific synapsin promoter were constructed as indicated in Fig. 2. Each transfer vector was transfected into HEK293T cells with the three supporting plasmids as described [5] to generate viruses. Viruses produced were concentrated by centrifugation and stored in 10% sucrose/PBS at −80 °C. ELISA for p24 determined virus particle concentration.

Cortical cultures were exposed to either vehicle control (no virus) or the indicated lentivirus at 10DIV. Lentivirus (87 ng per well of 24-well plate) was added to the cultures in 200 μl medium and gently rocked every 15 min for 1 h. After 1 h, 300 μl media was added to the cultures.

Treatments and cell survival analyses: Two days after infection, cells were exposed to glutamate excitotoxicity as follows. Media was removed and saved. Cells were washed once with warmed GBSS (Gey's Balanced Salt Solution). 500 μl of 300 μM glutamate was added in the presence or absence of the cannabinoid receptor agonist WIN55,212-2 (100 nM; Tocris), the cannabinoid receptor antagonist rimonabant (SR141716, 100 nM), or vehicle (DMSO). After 20 min at 37 °C, cells were washed once with GBSS, media replaced, and maintained at 37 °C. Each treatment was done in duplicate and repeated in three independent experiments. Twenty-four hours after the glutamate excitotoxic challenge, cell death in the cultures was determined using a LIVE/DEAD Viability/Cytotoxicity kit for mammalian cells (Invitrogen). Cells were washed twice with warmed GBSS and stained with 1 μM calceinAM and 0.8 μM ethidium homodimer-1 in GBSS at 26 °C for 20 min. Images of at least four randomly chosen fields of 0.136 mm^2 were captured and live and dead cells were counted using NIH ImageJ.