

# Cannabinoid Receptor–Interacting Protein 1a Modulates CB<sub>1</sub> Receptor Signaling and Regulation<sup>§</sup>

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Received October 21, 2014; accepted January 26, 2015

## ABSTRACT

Cannabinoid CB<sub>1</sub> receptors (CB<sub>1</sub>Rs) mediate the presynaptic effects of endocannabinoids in the central nervous system (CNS) and most behavioral effects of exogenous cannabinoids. Cannabinoid receptor–interacting protein 1a (CRIP<sub>1a</sub>) binds to the CB<sub>1</sub>R C-terminus and can attenuate constitutive CB<sub>1</sub>R-mediated inhibition of Ca<sup>2+</sup> channel activity. We now demonstrate cellular colocalization of CRIP<sub>1a</sub> at neuronal elements in the CNS and show that CRIP<sub>1a</sub> inhibits both constitutive and agonist-stimulated CB<sub>1</sub>R-mediated guanine nucleotide–binding regulatory protein (G-protein) activity. Stable overexpression of CRIP<sub>1a</sub> in human embryonic kidney (HEK)-293 cells stably expressing CB<sub>1</sub>Rs (CB<sub>1</sub>-HEK), or in N18TG2 cells endogenously expressing CB<sub>1</sub>Rs, decreased CB<sub>1</sub>R-mediated G-protein activation (measured by agonist-stimulated [<sup>35</sup>S]GTPγS [guanylyl-5'-[O-thio]-triphosphate] binding) in both cell lines and attenuated inverse agonism by rimonabant in CB<sub>1</sub>-HEK cells. Conversely, small-interfering RNA-mediated knockdown of CRIP<sub>1a</sub> in N18TG2 cells enhanced CB<sub>1</sub>R-mediated G-protein

activation. These effects were not attributable to differences in CB<sub>1</sub>R expression or endocannabinoid tone because CB<sub>1</sub>R levels did not differ between cell lines varying in CRIP<sub>1a</sub> expression, and endocannabinoid levels were undetectable (CB<sub>1</sub>-HEK) or unchanged (N18TG2) by CRIP<sub>1a</sub> overexpression. In CB<sub>1</sub>-HEK cells, 4-hour pretreatment with cannabinoid agonists downregulated CB<sub>1</sub>Rs and desensitized agonist-stimulated [<sup>35</sup>S]GTPγS binding. CRIP<sub>1a</sub> overexpression attenuated CB<sub>1</sub>R downregulation without altering CB<sub>1</sub>R desensitization. Finally, in cultured autaptic hippocampal neurons, CRIP<sub>1a</sub> overexpression attenuated both depolarization-induced suppression of excitation and inhibition of excitatory synaptic activity induced by exogenous application of cannabinoid but not by adenosine A1 agonists. These results confirm that CRIP<sub>1a</sub> inhibits constitutive CB<sub>1</sub>R activity and demonstrate that CRIP<sub>1a</sub> can also inhibit agonist-stimulated CB<sub>1</sub>R signaling and downregulation of CB<sub>1</sub>Rs. Thus, CRIP<sub>1a</sub> appears to act as a broad negative regulator of CB<sub>1</sub>R function.

## Introduction

Cannabinoid CB<sub>1</sub> receptors (CB<sub>1</sub>Rs) mediate most central nervous system (CNS) effects of the phytocannabinoid Δ<sup>9</sup>-tetrahydrocannabinol (THC) and the endocannabinoids (Howlett et al., 2002). CB<sub>1</sub>Rs are guanine nucleotide–binding regulatory protein (G-protein)–coupled receptors (GPCRs) that primarily

activate G<sub>i/o</sub> proteins (Howlett et al., 2002) and are widely distributed throughout the CNS (Herkenham et al., 1991). CB<sub>1</sub>Rs mediate synaptic plasticity via inhibition of neurotransmitter release (Kano et al., 2009) and regulate memory/cognition, motor activity, motivation, anxiety, appetite, and energy balance (Howlett et al., 2002). Thus, in addition to mediating abuse-related effects of cannabinoids, CB<sub>1</sub>Rs are attractive, albeit challenging, targets for drug discovery for the treatment of multiple CNS disorders (Pacher et al., 2006). However, prolonged CB<sub>1</sub>R activation by direct agonists produces tolerance, dependence, perturbation of transcription factors, and CB<sub>1</sub>R adaptation (Smith et al., 2010; Lazenka et al., 2013). Therefore, there is a need to better understand the regulation of CB<sub>1</sub>R signaling.

CB<sub>1</sub>Rs also interact with regulatory proteins that modulate CB<sub>1</sub>R function and mediate downstream signaling (Howlett et al., 2010; Smith et al., 2010). These proteins include such ubiquitous GPCR regulators as GPCR kinase-3 (GRK3) and β-arrestin2, which mediate CB<sub>1</sub>R desensitization and intracellular

This work was supported by the National Institutes of Health National Institute on Drug Abuse [R21-DA025321, R01-DA003690, R03-DA035424, F31-DA023747, F31-DA032215, R01-DA011322, K05-DA021696, and T32-DA007027], the National Institute on Neurologic Disorders and Stroke [T32-NS007288], the National Eye Institute [R21-EY021831], the Biotechnology and Biological Sciences Research Council [S19916], and the National Center for Advancing Translational Sciences [UL1-TR000058] in support of the A.D. Williams Fund of Virginia Commonwealth University. Mass spectrometry was supported in part with funding from the National Institute on Drug Abuse [P30-DA033934]. Rat microscopy was performed at the VCU Department of Anatomy and Neurobiology Microscopy Facility, supported in part with funding from the National Institute on Neurologic Disorders and Stroke [P30-NS047463].  
dx.doi.org/10.1124/mol.114.096495.

<sup>§</sup> This article has supplemental material available at molpharm.aspetjournals.org.

trafficking (Jin et al., 1999; Nguyen et al., 2012). Proteins that interact with a limited subset of receptor types include GPCR-associated sorting protein-1 (GASP1) and AP-3, which mediate CB<sub>1</sub>R targeting to lysosomes (Martini et al., 2007; Rozenfeld and Devi, 2008). In addition, CB<sub>1</sub>Rs interact with specific cannabinoid receptor-interacting proteins CRIP<sub>1a</sub> and CRIP<sub>1b</sub>, which are not known to interact with any other GPCR (Niehaus et al., 2007).

CRIP<sub>1a/b</sub> interact with the last nine amino acids of the CB<sub>1</sub>R C-terminus, but not with the CB<sub>2</sub>R (Niehaus et al., 2007). Both CRIP<sub>1a/b</sub> proteins are encoded by the *Cnr1* gene, which contains four exons: 1, 2, 3a, and 3b. Alternative splicing produces transcripts comprising exons 1, 2, and 3a (CRIP<sub>1a</sub>) or 1, 2, and 3b (CRIP<sub>1b</sub>). CRIP<sub>1a</sub> homologs are found throughout vertebrates, whereas CRIP<sub>1b</sub> appears to be limited to primates (Niehaus et al., 2007). The search for CB<sub>1</sub>R C-terminal-interacting proteins was initiated because this region exhibited autoinhibition of constitutive (agonist-independent) CB<sub>1</sub>R activity, which was relieved by truncation of the distal C-terminus of the receptor (Nie and Lewis, 2001a,b). Indeed, electrophysiological recordings in superior cervical ganglion (SCG) neurons showed that expression of CRIP<sub>1a</sub>, but not CRIP<sub>1b</sub>, attenuated constitutive CB<sub>1</sub>-mediated inhibition of calcium channels, revealed by elimination of the inverse agonist activity of rimonabant (SR141716A). However, coexpression of CRIP<sub>1a</sub> and CB<sub>1</sub>Rs did not alter agonist-induced inhibition of calcium currents or CB<sub>1</sub>R expression levels (Niehaus et al., 2007), suggesting that CRIP<sub>1a</sub> inhibits constitutive CB<sub>1</sub>R activity.

CRIP<sub>1a</sub> is highly expressed in the brain (Niehaus et al., 2007), and some reports suggest that CRIP<sub>1a</sub> is regulated by seizure activity. Sclerotic hippocampi from epileptic patients exhibited reduced expression of mRNA for both CRIP<sub>1a</sub> and CB<sub>1</sub>R (Ludanyi et al., 2008). In contrast, CRIP<sub>1a</sub> mRNA was elevated in rat hippocampus and cortex following kainic acid-induced seizures (Bojnik et al., 2012). These findings suggest CRIP<sub>1a</sub> involvement in modulating CB<sub>1</sub>R function in the pathogenesis or neuroadaptive response to epilepsy. Furthermore, CRIP<sub>1a</sub> expression inhibited the neuroprotective effects of a cannabinoid agonist and conferred a neuroprotective effect on an antagonist, in a cultured neuronal model of glutamate excitotoxicity (Stauffer et al., 2011). To date, evidence supports functional interactions between CRIP<sub>1a</sub> and CB<sub>1</sub>R in striatal GABAergic medium spiny neurons (Blume et al., 2013), glutamatergic hippocampal neurons (Ludanyi et al., 2008), and retinal presynaptic terminals (Hu et al., 2010). In addition, the *Cnr1* gene is hypermethylated in certain colorectal cancers (Lind et al., 2011; Oster et al., 2011), further suggesting potentially important functions of CRIP<sub>1a</sub> in multiple physiologic systems.

Despite the potential significance of CRIP<sub>1a</sub> as a novel player in the endocannabinoid system, relatively little is known about its function. The present study determined the effects of CRIP<sub>1a</sub> on constitutive and agonist-stimulated G-protein activation in CB<sub>1</sub>R-expressing cells. Because CRIP<sub>1a</sub> binds to the CB<sub>1</sub>R C-terminus, which interacts with regulatory proteins that mediate CB<sub>1</sub>R desensitization and downregulation, the effects of CRIP<sub>1a</sub> on prolonged agonist-induced adaptation in CB<sub>1</sub>R expression and signaling were also examined. To examine colocalization of CRIP<sub>1a</sub> with CB<sub>1</sub>Rs in a defined neuronal population in the CNS, colabeling studies were conducted in the cerebellum because both proteins are highly expressed in this region (Herkenham et al., 1991; Niehaus et al., 2007) and it plays a major role in cannabinoid dependence (Tzavara et al., 2000). Finally, to investigate the effects of CRIP<sub>1a</sub> on endocannabinoid function, its influence on depolarization-induced suppression of excitation (DSE) was examined in autaptic hippocampal neurons.

## Materials and Methods

### Chemicals

[<sup>35</sup>S]GTPγS (guanylyl-5'-[O-thio]-triphosphate; 1150–1300 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). [<sup>3</sup>H]SR141716A (44.0 Ci/mmol) was purchased from GE Healthcare (Buckinghamshire, UK). WIN55,212-2 [(3*R*)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone] (dissolved in ethanol), GDP, pertussis toxin, phenylmethanesulfonyl fluoride, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). THC, CP55,940 [(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol], levonantradol [(6*S*,6*αR*,9*R*,10*αR*)-9-hydroxy-6-methyl-3-[(2*R*)-5-phenylpentan-2-yl]oxy-5,6,6*a*,7,8,9,10,10*α*-octahydrophenanthridin-1-yl] acetate], HU-210 [3-(1,1'-dimethylheptyl)-6*αR*,7,10,10*αR*-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[ $\beta$ , $\delta$ ]pyran-9-methanol], noladin ether, and SR141716A [5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide] were provided as solutions in ethanol by the Drug Supply Program of the National Institute on Drug Abuse (NIDA, Rockville, MD). Methanandamide was purchased from Cayman Chemical (Ann Arbor, MI). LI-COR Odyssey infrared dye secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE).  $\alpha$ -Tubulin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). All other reagent-grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Stable Transfection and Treatment of Cultured Cells.** Human embryonic kidney (HEK) 293 cells stably expressing the human CB<sub>1</sub>R subcloned into pcDNA3 vector (hCB<sub>1</sub>-HEK) (Abood et al., 1997) were cultured in Dulbecco's modified Eagle's medium (DMEM), 1× high glucose containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin (P/S), 0.25 mg/ml geneticin (G418), and 15 mM HEPES. hCB<sub>1</sub>-HEK cells stably cotransfected with CRIP<sub>1a</sub> subcloned into the pcDNA3.1zeo vector (hCB<sub>1</sub>-HEK-CRIP<sub>1a</sub>)

**ABBREVIATIONS:** 2-AG, 2-arachidonoylglycerol; 2-AGE, 2-arachidonoylglycerol ether; AEA, arachidonylethanolamide; ANOVA, analysis of variance; BSA, bovine serum albumin; CB<sub>1</sub>R, cannabinoid CB<sub>1</sub> receptor; CNS, central nervous system; CP55,940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; CPA, cyclopentyladenosine; DMEM, Dulbecco's modified Eagle's medium; DSE, depolarization-induced suppression of excitation; EPSC, excitatory postsynaptic current; FBS, fetal bovine serum; G418, geneticin; GPCR, G-protein-coupled receptor; GST, glutathione S-transferase; GTPγS, guanylyl-5'-[O-thio]-triphosphate; HA, hemagglutinin; HEK, human embryonic kidney; HU-210, 3-(1,1'-dimethylheptyl)-6*αR*,7,10,10*αR*-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[ $\beta$ , $\delta$ ]pyran-9-methanol; ir, immunoreactivity; levonantradol, [(6*S*,6*αR*,9*R*,10*αR*)-9-hydroxy-6-methyl-3-[(2*R*)-5-phenylpentan-2-yl]oxy-5,6,6*a*,7,8,9,10,10*α*-octahydrophenanthridin-1-yl] acetate; MAEA, methanandamide; PBS, phosphate-buffered saline; P/S, penicillin/streptomycin; PTX, pertussis toxin; SCG, superior cervical ganglion; siRNA, small-interfering RNA; SR141716A, rimonabant, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; SV2, synaptic vesicle 2; THC,  $\Delta^9$ -tetrahydrocannabinol; WIN55,212-2, [(3*R*)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone.

(Niehaus et al., 2007) were cultured in the same media with the addition of 0.1 mg/ml zeocin.

Stable CRIP<sub>1a</sub>-overexpression and -knockdown N18TG2 cell clones were generated by transfecting (Lipofectamine 2000; Invitrogen, Carlsbad, CA) N18TG2 cells with either a pcDNA3.1-CRIP<sub>1a</sub> mouse cDNA plasmid for overexpression, or two different pRNATin-H1.2 small-interfering RNA (siRNA)-CRIP<sub>1a</sub> vectors for knockdown. The GenScript siRNA target finder program (GenScript, Piscataway, NJ) was used to select CRIP<sub>1a</sub> siRNA-target sequences. CRIP<sub>1a</sub> N18TG2 cell lines were generated by isolating and expanding G418-resistant single colonies in selection media containing 600 µg/ml G418 (Gibco/Life Technologies, Grand Island, NY). Cells were maintained in DMEM/F12 media with 10% heat-inactivated bovine serum, Gluta-Max, and P/S, with 0.25 mg/ml geneticin.

For ligand pretreatments, appropriate concentrations of drugs were added to treatment media (DMEM, 1% FBS, P/S) and sterile-filtered, and drug treatment media was added to cells for the appropriate time period. To terminate drug treatments, cells were rinsed twice for 2 minutes with warm rinse media (DMEM, 1% FBS), and harvested for assays.

### Membrane Homogenate Preparation

Cells were harvested in phosphate-buffered saline with 0.4% (w/v) EDTA or by gentle scraping and centrifuged at 1000g for 10 minutes to remove media. Cells were homogenized in ice-cold 50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, and 1 mM EGTA, pH 7.4 (membrane buffer), and centrifuged at 50,000g for 10 minutes. The resulting pellets were homogenized in 50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 0.2 mM EGTA, pH 7.4 (TME buffer) with 100 mM NaCl, and protein content was determined.

Cerebella were obtained from adult male Sprague-Dawley rats (Harlan, Indianapolis, IN). Rats were sacrificed by rapid decapitation, brains were removed, and cerebella were dissected on ice. Cerebellum samples were homogenized in membrane buffer and membranes were isolated by centrifugation as described above. Experiments were performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, 7th Edition.

### CRIP<sub>1a</sub> Generation, Purification, and Determination of Stoichiometry

A CRIP<sub>1a</sub> cDNA insert was subcloned into the BamHI and XhoI sites of the pGEX-4T-1 vector (GE Healthcare, Piscataway, NJ) to generate a glutathione *S*-transferase (GST)-tagged CRIP<sub>1a</sub> (GST tag-thrombin cleavage site-CRIP<sub>1a</sub>) construct. Plasmid DNA encoding GST-tagged CRIP<sub>1a</sub> was transformed into *Escherichia coli* BL21-DE3-competent cells. *E. coli* were grown to optical density 600 = 0.6 from a single colony and then GST-tagged CRIP<sub>1a</sub> expression was induced via addition of isopropyl thiogalactoside (1 mM) for 6 hours. *E. coli* were collected via centrifugation (1000g, 10 minutes, 4°C) and a bacterial lysate produced via sonication with lysozyme (25 µg/ml). CRIP<sub>1a</sub> induction and solubility tests were performed by polyacrylamide gel electrophoresis on harvested lysates using 10% polyacrylamide gels, which were stained with Coomassie blue to verify protein expression. Crude lysate was then separated into soluble and insoluble lysates. GST-tagged CRIP<sub>1a</sub> was isolated from bacterial lysate using a GSTrap FF column (Amersham Biosciences, Piscataway, NJ) as follows. The column was equilibrated with binding buffer [0.1 M phosphate buffered saline (PBS)], bacterial lysate was added to allow GST-CRIP binding, the column was washed (PBS), and the GST tag was cleaved via thrombin (500 units in 0.5 ml PBS). Following elution with PBS, CRIP<sub>1a</sub> was purified by the subsequent removal of thrombin using HiTrap benzamidine column purification. Briefly, the column was equilibrated with binding buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4) and then the sample was added to the column followed by elution with binding buffer. CRIP<sub>1a</sub> eluates were collected and pooled, and CRIP<sub>1a</sub> pools and a BSA protein standard curve were subjected to polyacrylamide gel

electrophoresis using 15% polyacrylamide gels, and visualized by Coomassie blue stain. Stained gel images were captured via ImageJ, and CRIP<sub>1a</sub> concentration was determined by subsequent linear regression analysis (Windows Excel). Purified CRIP<sub>1a</sub> concentration curves were then generated in tandem with hCB<sub>1</sub>-HEK (±CRIP<sub>1a</sub>) cell membrane preparations or rat cerebellar membranes to determine CRIP<sub>1a</sub> concentration via immunoblot analysis on 15% polyacrylamide gels. From these data, the stoichiometric relationship between CRIP<sub>1a</sub> concentration in cell membranes and CB<sub>1</sub>R levels, determined by [<sup>3</sup>H]SR141716A *B*<sub>max</sub> values, was calculated.

### Immunoblotting

Samples (70 µg) of cell membrane homogenates or purified CRIP<sub>1a</sub> standards were added to sample buffer (1 M Tris-HCl, 20% SDS, 1 M dithiothreitol, 60% sucrose, bromophenol blue) and boiled for 10 minutes. Samples were loaded into 15% SDS polyacrylamide gels, and electrophoresis was conducted at 120 V for 1.5 hours. Proteins were transferred by electrophoresis onto polyvinylidene difluoride membranes at 70 V for 70 minutes. Blots were blocked for 1 hour at room temperature with 5% (w/v) nonfat dry milk and then rinsed with Tris-buffered saline with 0.1% (v/v) Tween-20 (TBST). Primary antibody (rabbit anti-CRIP<sub>1a</sub> antiserum 077.4; 1:500; Niehaus et al., 2007) was incubated overnight at 4°C, followed by TBST rinse. Secondary antibody (LI-COR goat anti-rabbit 800 CW IR dye, 1:5,000) was then incubated at room temperature for 1 hour, followed by TBST rinse. Blots were visualized with the LI-COR Odyssey system.

### [<sup>3</sup>H]SR141716A Binding

Saturation analysis of [<sup>3</sup>H]SR141716A binding was performed by incubating 30 µg of membrane protein with 0.5–10 nM [<sup>3</sup>H]SR141716A in TME with 0.5% (w/v) BSA, in a total volume of 0.5 ml with and without 5 µM unlabeled SR141716A to determine nonspecific binding. The assay was incubated for 90 minutes at 30°C and terminated by vacuum filtration through GF/B glass fiber filters that were presoaked in Tris buffer containing 0.5% (w/v) BSA. Bound radioactivity was determined using liquid scintillation spectrophotometry at 45% efficiency for [<sup>3</sup>H].

### [<sup>3</sup>H]CP55,940 Binding

Saturation analysis of [<sup>3</sup>H]CP55,940 binding was performed by incubating 100 µg of membrane protein with 0.2–8 nM [<sup>3</sup>H]CP55,940 in TME (without NaCl) with 0.5% (w/v) BSA, in a total volume of 0.5 ml with and without 5 µM unlabeled SR141716A to determine nonspecific binding. The assay was incubated for 90 minutes at 30°C and terminated by vacuum filtration through GF/B glass fiber filters that were presoaked in Tris buffer containing 0.5% (w/v) BSA. Bound radioactivity was determined using liquid scintillation spectrophotometry at 45% efficiency for [<sup>3</sup>H].

### [<sup>35</sup>S]GTPγS Binding

Cell membrane preparations (10 µg of protein) were incubated with various drugs, 100 mM NaCl, 0.1% BSA, 10 µM (CB<sub>1</sub>-HEK), or 20 µM (N18TG2) GDP and 0.1 nM [<sup>35</sup>S]GTPγS in TME in 0.5-ml total volume, for 2 hours at 30°C. In some experimental conditions, 100 mM NaCl was omitted to increase constitutive receptor activity. Basal binding was assessed in the absence of agonist, and nonspecific binding was measured with 10-µM unlabeled GTPγS. The reaction was terminated by vacuum filtration through GF/B glass fiber filters. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for [<sup>35</sup>S].

### Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry Analysis of Endocannabinoids

Arachidonylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) were measured using a method modified from Di Marzo et al. (2000).

Briefly, 2 pmol of AEA-*d*<sub>8</sub> and 2 nmol 2AG-*d*<sub>8</sub> as deuterated internal standards were added to each sample. The endocannabinoids were extracted from the samples with 3 volumes chloroform/methanol (2:1, v/v) containing 34.8 mg phenylmethanesulfonyl fluoride/ml and a 0.73% (w/v) sodium chloride mixture. The organic phases from the three extractions were pooled and the organic solvents were evaporated to dryness with nitrogen. Dried samples were reconstituted in 100  $\mu$ l of chloroform and mixed with 1 ml of cold acetone to precipitate proteins. The mixtures were centrifuged and the upper layers were collected and evaporated to dryness with nitrogen. The extracts were reconstituted with 100  $\mu$ l of methanol and placed in autosample vials for liquid chromatography–electrospray ionization–tandem mass spectrometry analysis. The AEA and 2-AG were separated and detected using a Shimadzu SCL HPLC system (Kyoto, Japan) with a Discovery HS C18 Column 15 cm  $\times$  2.1 mm, 3  $\mu$ m (Supelco/Sigma-Aldrich, Bellefonte, PA) kept at 40°C and an Applied Biosystems 3200 Q trap with a turbo V source for TurbolonSpray (Ontario, Canada) run in multiple reaction monitoring mode. The mobile phase consisted of 10:90 water/methanol with 0.1% (w/v) ammonium acetate and 0.1% (v/v) formic acid. The flow rate was 0.3 ml/min and total run time was 10.00 minutes. The injection volume was 20  $\mu$ l and the autosampler temperature was set at 5°C. The mass spectrometer was run in electrospray ionization positive mode. Ions were analyzed in multiple reaction monitoring mode and the following transitions were monitored: (348 > 62) and (348 > 91) for AEA; (356 > 62) for AEA-*d*<sub>8</sub>; (379 > 287) and (379 > 269) for 2-AG; (387 > 96) for 2AG-*d*<sub>8</sub>. The standard curves for the samples were 0.039–1.25 pmol AEA and 0.06–2.0 nmol 2-AG. The limit of detection and limit of quantification were set at 0.039 pmol for AEA and 0.063 nmol for 2-AG.

### Hippocampal Culture Preparation

All procedures used in this study were approved by the Animal Care Committee of Indiana University and conform to the Guidelines of the National Institutes of Health on the Care and Use of Animals. Mouse (CD1 strain) hippocampal neurons isolated from the CA1–CA3 region were cultured on microislands as described previously (Furshpan et al., 1976; Bekkers and Stevens, 1991). Neurons were obtained from animals (age postnatal day 0–2) and plated onto a feeder layer of hippocampal astrocytes that had been laid down previously (Levison and McCarthy, 1991). Cultures were grown in high glucose (20 mM) DMEM containing 10% horse serum, without mitotic inhibitors and used for recordings after 8 days in culture and for no more than 3 hours after removal from culture medium.

### Electrophysiology

When a single neuron is grown on a small island of permissive substrate, it forms synapses—or “autapses”—onto itself. All experiments were performed on isolated autaptic neurons. Whole cell voltage-clamp recordings from autaptic neurons were carried out at room temperature using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA). The extracellular solution contained (in mM) 119 NaCl, 5 KCl, 2.5 CaCl<sub>2</sub>, 1.5 MgCl<sub>2</sub>, 30 glucose, and 20 HEPES. Continuous flow of solution through the bath chamber (~2 ml/min) ensured rapid drug application and clearance. Drugs were typically prepared as stocks, and then diluted into extracellular solution at their final concentration and used on the same day.

Recording pipettes of 1.8–3 M $\Omega$  were filled with (in mM) 121.5 potassium gluconate, 17.5 KCl, 9 NaCl, 1 MgCl<sub>2</sub>, 10 HEPES, 0.2 EGTA, 2 MgATP, and 0.5 LiGTP. Access resistance and holding current were monitored and only cells with both stable access resistance and holding current were included for data analysis. Conventional stimulus protocol: The membrane potential was held at –70 mV and excitatory postsynaptic currents (EPSCs) were evoked every 20 seconds by triggering an unclamped action current with a 1.0-millisecond depolarizing step. The resultant evoked waveform consisted of a brief stimulus artifact and a large downward spike representing inward sodium currents, followed by the slower EPSC. The size of the recorded EPSCs was calculated by integrating the evoked current to yield a charge value

(in pC). Calculating the charge value in this manner yields an indirect measure of the amount of neurotransmitter released, at the same time minimizing the effects of cable distortion on currents generated far from the site of the recording electrode (the soma). Data were acquired at a sampling rate of 5 kHz.

**DSE Stimuli.** After establishing a 10- to 20-second 0.5 Hz baseline, DSE was evoked by depolarizing to 0 mV for 50 milliseconds, 100 milliseconds, 300 milliseconds, 500 milliseconds, 1 second, 3 seconds, and 10 seconds, followed in each case by resumption of a 0.5-Hz stimulus protocol for 20–80+ seconds, allowing EPSCs to recover to baseline values. This approach allowed us to determine the sensitivity of the synapses to DSE induction.

### Transfection of Autaptic Cultures

Neurons were transfected using a modified calcium phosphate–based method (Jiang et al., 2004). Briefly, plasmids for hemagglutinin (HA)-tagged CRIP<sub>1a</sub> and for the fluorescent marker mCherry (2  $\mu$ g/well) were combined with 2 M CaCl<sub>2</sub> and gradually added to HEPES-buffered saline; the mixture was added to the serum-free neuronal media. Coverslips were incubated with this mixture in a separate well for 2.5 hours and extra media was placed in a 10% CO<sub>2</sub> incubator to induce equilibration. At the end of 2.5 hours, the reaction mixture was replaced with acidified serum-free media for 20 minutes. After this, cells were returned to their home wells.

### Immunostaining of Autaptic Cultures

Autaptic neurons cultured on coverslips were transfected and prepared as described previously (Straiker et al., 2009). Briefly, paraformaldehyde-fixed neurons were incubated with an HA11 antibody overnight at 4°C and then washed six times with 0.1 M PBS. Cells were next incubated with fluorescein isothiocyanate–conjugated donkey secondary antibody (anti-mouse, 1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1.5 hours at room temperature. Finally coverslips were washed, dried, and mounted. Images were acquired with a Leica TCS SP5 confocal microscope (Leica Microsystems) using Leica LAS AF software and a 63 $\times$  oil objective. Images were processed using ImageJ (available at <http://rsbweb.nih.gov/ij/>) and/or Photoshop (Adobe Inc., San Jose, CA).

### Immunostaining of Rat Brain Sections

Tissue preparation and immunofluorescence labeling were conducted as described (Falenski et al., 2007) with minor modification. Adult male Sprague-Dawley rats (200–250 g) (Harlan) were housed on a 12-hour light/dark cycle in single cages and were provided with food and water ad libitum. Rats were injected with ketamine/xylazine (75 mg/kg, i.p.), flushed transcardially with saline, and perfused with 4% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.4). Brains were removed and postfixed in the same fixative overnight, and then placed in sodium phosphate buffer plus 30% sucrose for cryoprotection. Coronal sections of the cerebellum (20  $\mu$ m) were cut on a cryostat maintained at –20°C and thaw-mounted onto gelatin-subbed slides. Sections were incubated for 30 minutes in PBS containing 0.1% Triton  $\times$  100 and then for 1 hour in SuperBlock Blocking Buffer (Pierce, Rockford, IL) with 0.1% Triton  $\times$  100. Sections were then incubated with rabbit anti-CRIP<sub>1a</sub>-Ct (antiserum 077.4, 1:1000) and guinea pig anti-CB<sub>1</sub>R-Ct (against CB<sub>1</sub>R residues 401–473, 1:1000) in SuperBlock Blocking Buffer for 72 hours, followed by incubation in appropriate secondary antibodies (CRIP<sub>1a</sub>, Alexa-488; CB<sub>1</sub>, Alexa-595; 1:200) for 1 hour. Slides were washed, and coverslips were mounted using Vectashield (Vector, Burlingame, CA) and sealed with clear nail polish. Images were captured at 60 $\times$  magnification on a Zeiss 700 laser scanning confocal microscope.

For immunolabeling of CRIP<sub>1a</sub> with visualization by immunohistochemistry, brains were removed and postfixed in Bouin's fixative for 3 days at room temperature before being embedded in paraffin wax. Coronal sections (10  $\mu$ m) were cut and mounted on glass slides.

Dewaxed sections were blocked with 5% normal goat serum/PBS with 0.2% Triton X-100 (PBST) and then incubated with 077.4 CRIP<sub>1a</sub> antiserum diluted 1:1000 in 5% normal goat serum in PBST. Bound antibodies were revealed by using the avidin-biotin complex, peroxidase method (Vector Laboratories). The specificity of immunostaining was established by testing antisera preabsorbed with the KPNETRSMLWVN-KESFL peptide antigen (20  $\mu$ M), which comprises the C-terminal region of the rat CRIP<sub>1a</sub> protein.

### Immunostaining of Mouse Brain Sections

GAD67-GFP mice were generated by Dr. Yuchio Yanagawa [Gunma University, Gunma, Japan (Tamamaki et al., 2003)]. Brain sections were prepared from mice perfused with 4% paraformaldehyde. Brains were removed and immersed in 30% sucrose for 24–72 hours at 4°C. Tissue was then frozen in a freezing compound (Tissue-Tek O.C.T., VWR, Radnor, PA) and sectioned (15–30  $\mu$ m) using a Leica CM1850 cryostat (Leica Microsystems). Tissue sections were mounted onto Superfrost Plus slides, washed in PBS, then treated with SEA BLOCK blocking buffer (Thermo Scientific, Rockford, IL). Cells were treated overnight at 4°C with antibodies prepared in PBS and detergent (saponin, 0.1%). Secondary antibodies (Alexa 488, 495, or 647, anti-mouse, anti-rabbit, or anti-guinea pig as appropriate; Invitrogen, Carlsbad, CA) were subsequently applied overnight at 4°C. Monoclonal synaptic vesicle 2 (SV2) and GAD65 antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA) and used at 1:500. The guinea pig CB<sub>1</sub> and CRIP<sub>1a</sub> antibodies were developed in house and used at 1:300 and have been described previously (Berghuis et al., 2007; Hu et al., 2010). Images were acquired with a Leica TCS SP5 confocal microscope (Leica Microsystems) using Leica LAS AF software and a 63 $\times$  oil objective. Images were processed using ImageJ (available at <http://rsbweb.nih.gov/ij/>) and/or Photoshop (Adobe Inc., San Jose, CA). Images were modified only in terms of brightness and contrast.

### Data Analysis

Unless otherwise noted, all binding data are reported as mean values  $\pm$  standard error of the mean (S.E.M.) of at least three independent experiments performed in duplicate (<sup>3</sup>H]SR141716A and [<sup>3</sup>H]CP55,940) or triplicate (<sup>35</sup>S]GTP $\gamma$ S). Data were analyzed using GraphPad/Prism v5.0 software (GraphPad Software, La Jolla, CA).  $B_{\max}$ ,  $K_D$ ,  $E_{\max}$ , and  $EC_{50}$  values were determined by nonlinear regression analysis. Non-linear regression was used to fit the data to the following equation:  $B = (B_{\max})(L)/(K_D + L)$ , where B is the amount of <sup>3</sup>H-ligand bound at each ligand concentration L,  $B_{\max}$  is the maximal predicted amount of <sup>3</sup>H-ligand bound, and  $K_D$  is the equilibrium dissociation constant for <sup>3</sup>H-ligand binding. Saturation curve-fitting analysis for [<sup>3</sup>H]SR141716A and [<sup>3</sup>H]CP55,940 were weighted by  $1/x$  ( $1/x^2$  H-ligand concentration), because nonspecific binding is relatively high for these cannabinoid ligands and increases linearly with <sup>3</sup>H-ligand concentration. For studies of G-protein activation,  $E_{\max}$  and  $\log EC_{50}$  were likewise

determined from log concentration-effect curves, where  $E$  is the percentage change in [<sup>35</sup>S]GTP $\gamma$ S binding relative to basal binding at any given concentration of receptor ligand,  $E_{\max}$  is the maximal percentage change from basal [<sup>35</sup>S]GTP $\gamma$ S binding observed at maximally effective concentrations of ligand, and  $\log EC_{50}$  is the  $\log^{10}$  of the molar concentration of receptor ligand producing half-maximal modulation of [<sup>35</sup>S]GTP $\gamma$ S binding. Statistical comparison was performed on  $\log EC_{50}$  values, which were then transformed and reported as  $EC_{50}$  values. Basal [<sup>35</sup>S]GTP $\gamma$ S binding is determined in the absence of receptor ligand. Net stimulated [<sup>35</sup>S]GTP $\gamma$ S binding is defined as agonist-stimulated minus basal binding. Percentage stimulation is defined as (net stimulated binding/basal binding)  $\times$  100%.

Significance was determined using analysis of variance (ANOVA) and the posthoc Newman-Keuls multiple comparison test for comparison of three or more conditions or by Student's  $t$  test for comparison of two conditions. In the few instances where unequal variance between groups was detected by  $F$  test, Welch's correction for unequal variance was applied. Two-way ANOVA and the posthoc Bonferroni test were used in experiments comparing two or more sets of independent variables. Results were considered statistically significant when the  $P$  value was  $\leq 0.05$ . All inferential statistics were performed using GraphPad Prism v5.0d software.

## Results

**Stoichiometry of CB<sub>1</sub>R and CRIP<sub>1a</sub> Expression in Stably Transfected HEK-293 Cells and Rat Cerebellum.** Niehaus et al. (2007) previously showed that CRIP<sub>1a</sub> localizes to the cell membrane and interacts with the C-terminal tail of CB<sub>1</sub>Rs without affecting CB<sub>1</sub>R expression levels. To confirm that stable coexpression of CRIP<sub>1a</sub> did not affect CB<sub>1</sub>R expression and to determine CRIP<sub>1a</sub>/CB<sub>1</sub> expression ratios, CB<sub>1</sub>R  $B_{\max}$  values were obtained using [<sup>3</sup>H]SR141716A saturation binding analysis (Supplemental Fig. 1) in HEK-293 cells stably expressing CB<sub>1</sub>Rs (CB<sub>1</sub>-HEK) and the same CB<sub>1</sub>-HEK cell line was then stably transfected with CRIP<sub>1a</sub> (CB<sub>1</sub>-HEK-CRIP<sub>1a</sub>). Results showed no significant difference in CB<sub>1</sub>R expression as determined by [<sup>3</sup>H]SR141716A  $B_{\max}$  values between CB<sub>1</sub>-HEK cells with and without stable coexpression of CRIP<sub>1a</sub> (Table 1). Likewise, no difference in the  $K_D$  value of [<sup>3</sup>H]SR141716A was observed between CB<sub>1</sub>-HEK and CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cell lines (Table 1). These results confirm that stable CRIP<sub>1a</sub> overexpression did not affect CB<sub>1</sub>R expression or affinity for [<sup>3</sup>H]SR141716A in CB<sub>1</sub>-HEK cells.

The effect of CRIP<sub>1a</sub> on CB<sub>1</sub>R function can probably be determined in part by the molar ratio of CRIP<sub>1a</sub> to CB<sub>1</sub>R. To determine the stoichiometric relationship of CRIP<sub>1a</sub> to CB<sub>1</sub>R expression, quantitative immunoblot analysis of CRIP<sub>1a</sub> was

TABLE 1  
Stoichiometry of CRIP<sub>1a</sub> and CB<sub>1</sub> receptor expression in CB<sub>1</sub>-HEK and CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells compared with rat cerebellum

Membranes prepared from the indicated tissue sources were incubated with varying concentrations of [<sup>3</sup>H]SR141716A, as described in *Materials and Methods*.  $B_{\max}$  and  $K_D$  values were derived from nonlinear regression analysis of the saturation binding curves. CRIP<sub>1a</sub> protein values were determined by quantitative immunoblot of the indicated tissue source, using purified CRIP<sub>1a</sub> as an internal standard, as described in *Materials and Methods*. Data are mean values  $\pm$  S.E.M. ( $n = 4-6$ )

Tissue Source	CB <sub>1</sub> $B_{\max}$ <i>pmol/mg</i>	CB <sub>1</sub> $K_D$ <i>nM</i>	CRIP <sub>1a</sub> <i>pmol/mg</i>	Molar Ratio CRIP <sub>1a</sub> /CB <sub>1</sub>
CB <sub>1</sub> -HEK	1.34 $\pm$ 0.12	1.45 $\pm$ 0.19	0.56 $\pm$ 0.13	0.42 $\pm$ 0.09
CB <sub>1</sub> -HEK-CRIP <sub>1a</sub>	1.17 $\pm$ 0.13	1.63 $\pm$ 0.35	8.20 $\pm$ 0.64**	7.01 $\pm$ 0.55**
Rat cerebellum	3.76 $\pm$ 0.31	0.49 $\pm$ 0.04	115 $\pm$ 12.2	32.02 $\pm$ 4.39

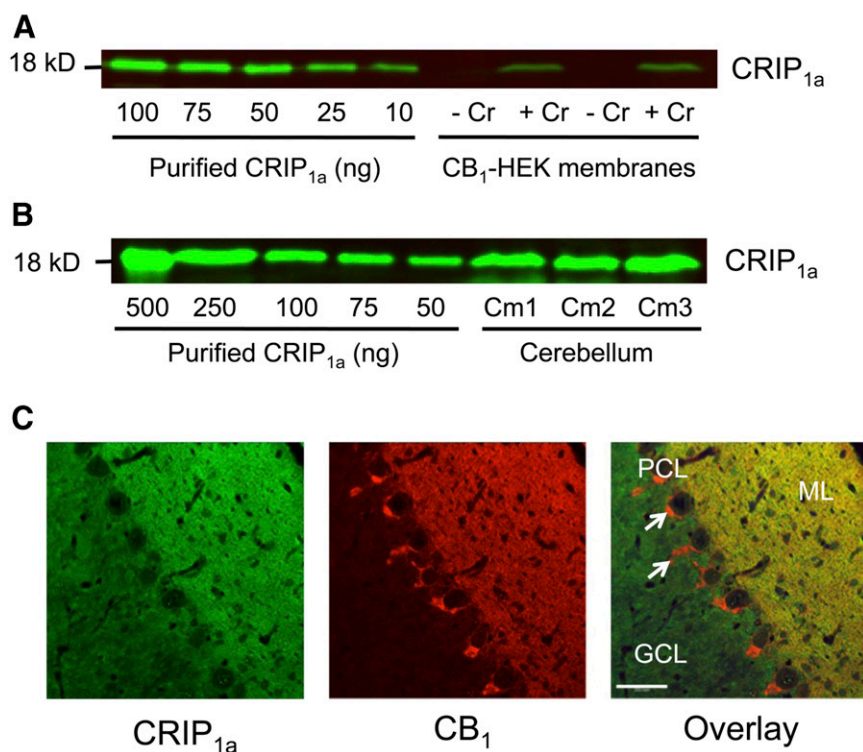
\*\* $P < 0.01$  different from corresponding value in CB<sub>1</sub>-HEK cells by Student's  $t$  test with Welch's correction (note: values from cerebellum were not included in the analysis).

performed. CRIP<sub>1a</sub> was purified using GST-pull-down methodology and CRIP<sub>1a</sub> concentration curves were included in immunoblots to determine unknown CRIP<sub>1a</sub> concentrations (Fig. 1A). The C-terminally directed CRIP<sub>1a</sub> antibody (Niehaus et al., 2007) produced relatively clean blots, with few or no extraneous labeling other than the 18-kDa band corresponding to CRIP<sub>1a</sub> (Supplemental Fig. 2). Experimentally determined CRIP<sub>1a</sub> concentrations were then compared with CB<sub>1</sub>R  $B_{max}$  values to determine the molar stoichiometric relationship of CRIP<sub>1a</sub>/CB<sub>1</sub>Rs (Table 1). In CB<sub>1</sub>-HEK cells, the molar ratio of CRIP<sub>1a</sub>/CB<sub>1</sub> was less than 1 ( $0.34 \pm 0.08$ ), indicating that the CB<sub>1</sub>R is in molar excess relative to CRIP<sub>1a</sub> natively expressed in CB<sub>1</sub>-HEK cells. In CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells, CRIP<sub>1a</sub> was in molar excess to the CB<sub>1</sub>R, with a CRIP<sub>1a</sub>/CB<sub>1</sub>R ratio of  $5.44 \pm 0.42$ , which was significantly different from CB<sub>1</sub>-HEK cells. For comparison of expression ratios of CRIP<sub>1a</sub>/CB<sub>1</sub>R in a native tissue, [<sup>3</sup>H]SR141716A saturation analysis and quantitative immunoblotting of CRIP<sub>1a</sub> were also conducted in rat cerebellar membranes (Table 1). Interestingly, rat cerebellum had a CRIP<sub>1a</sub>/CB<sub>1</sub>R molar ratio of  $32.02 \pm 4.39$ , indicating a greater molar excess of CRIP<sub>1a</sub> relative to the CB<sub>1</sub>R than in the CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells. These results demonstrate that membranes from CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells express a significantly greater molar ratio of CRIP<sub>1a</sub>/CB<sub>1</sub>R than CB<sub>1</sub>-HEK cells, but not greater than the ratio obtained in membranes from rat cerebellar homogenates.

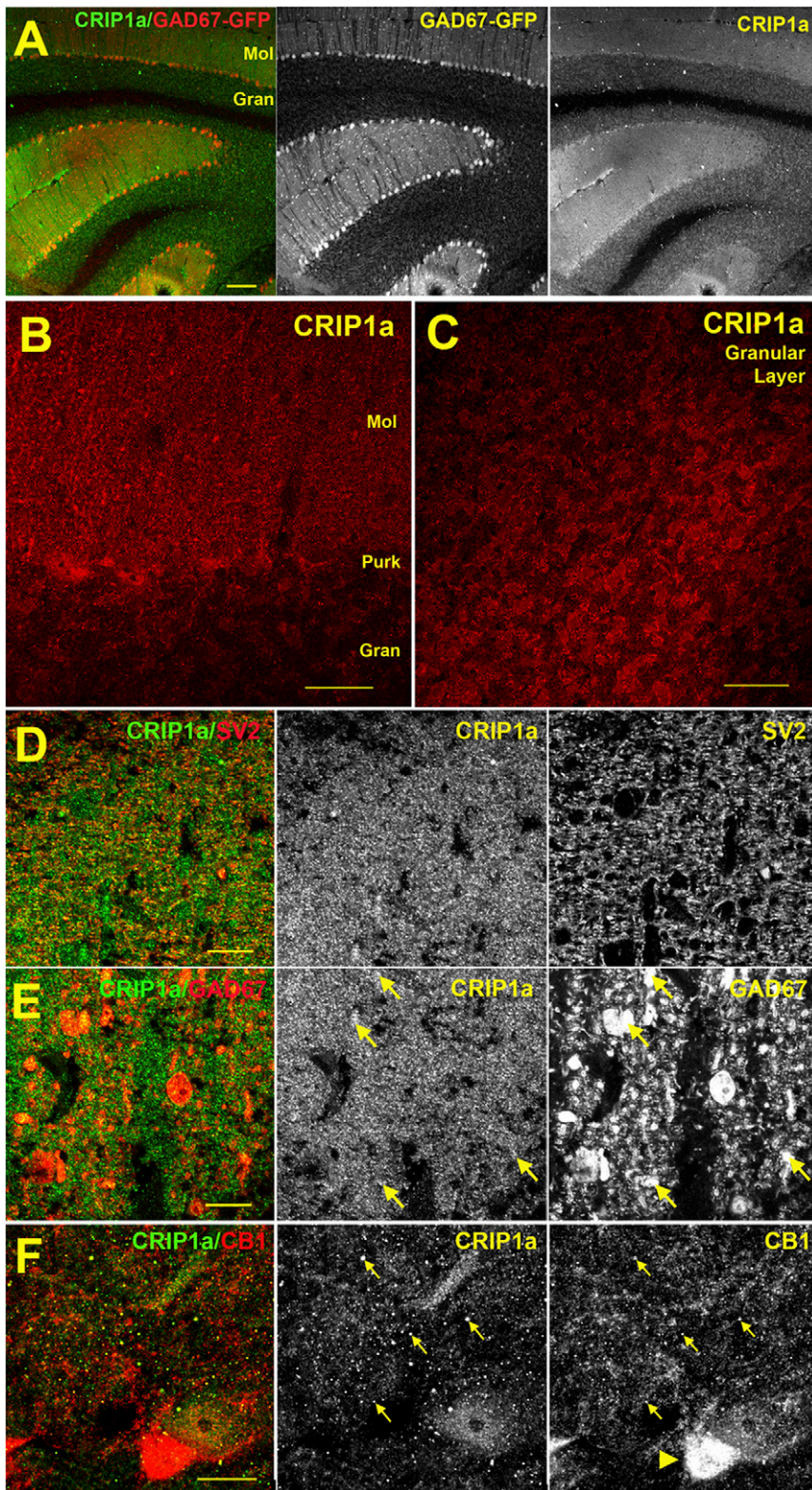
The stoichiometry result in rat cerebellum is complicated by the question of whether CRIP<sub>1a</sub> is colocalized in the same cells with CB<sub>1</sub>Rs in this tissue. Therefore, to determine whether CB<sub>1</sub>Rs and CRIP<sub>1a</sub> are colocalized in rat cerebellum, brain sections were colabeled with rabbit anti-CRIP<sub>1a</sub> (green) and guinea pig anti-CB<sub>1</sub>R-Ct (red) antibodies (Fig. 1C). Colocalization at this level of resolution of CRIP<sub>1a</sub>-immunoreactivity

(ir) with CB<sub>1</sub>-ir is evident in the molecular layer. CRIP<sub>1a</sub>-ir is also evident at lower levels in the granule cell layer, where CB<sub>1</sub>-ir is seldom detected. In the Purkinje cell layer, intense CB<sub>1</sub>R-ir can be seen in putative axon terminals, possibly from basket cells that are presynaptic to the unstained somata of Purkinje cells; little or no CRIP<sub>1a</sub>-ir is evident in the axon terminals of basket cells. The widespread but heterogeneous distribution of CRIP<sub>1a</sub> among rat cerebellar layers was confirmed by immunohistochemical staining visualized with peroxidase labeling, which was blocked by coincubation with an antigen peptide corresponding to the C-terminal region of CRIP<sub>1a</sub> (Supplemental Fig. 3). These results indicate that in the cerebellum CRIP<sub>1a</sub> is putatively colocalized with CB<sub>1</sub>Rs in axonal fibers arising from the glutamatergic granule cells that project throughout the molecular layer, and the granule cell layer also contains CRIP<sub>1a</sub> at lower levels.

To determine the localization of CRIP<sub>1a</sub> and its colocalization with CB<sub>1</sub>Rs in specific cellular elements of the cerebellum, immunofluorescent labeling was performed in the GAD67-GFP mouse, which expresses green fluorescent protein in GABAergic neurons (Tamamaki et al., 2003), with costaining of multiple subcellular markers. Using an antibody developed against CRIP<sub>1a</sub> (Hu et al., 2010), we examined the distribution of this protein in multiple cerebellar subregions. CRIP<sub>1a</sub> is widely distributed in the cerebellum, abundant in both the molecular and granular layers (Fig. 2A-C), similar to what was detected in the rat using an independent antibody in Fig. 1. In the molecular layer the staining substantially overlaps with SV2, a presynaptic marker (Fig. 2D), consistent with a presynaptic localization. However, we also observed costaining with GAD67-GFP positive processes, perhaps belonging to Purkinje cells (Fig. 2E). CRIP<sub>1a</sub> was widely colocalized with CB<sub>1</sub>R throughout the molecular layer (Fig. 2F), but not in the



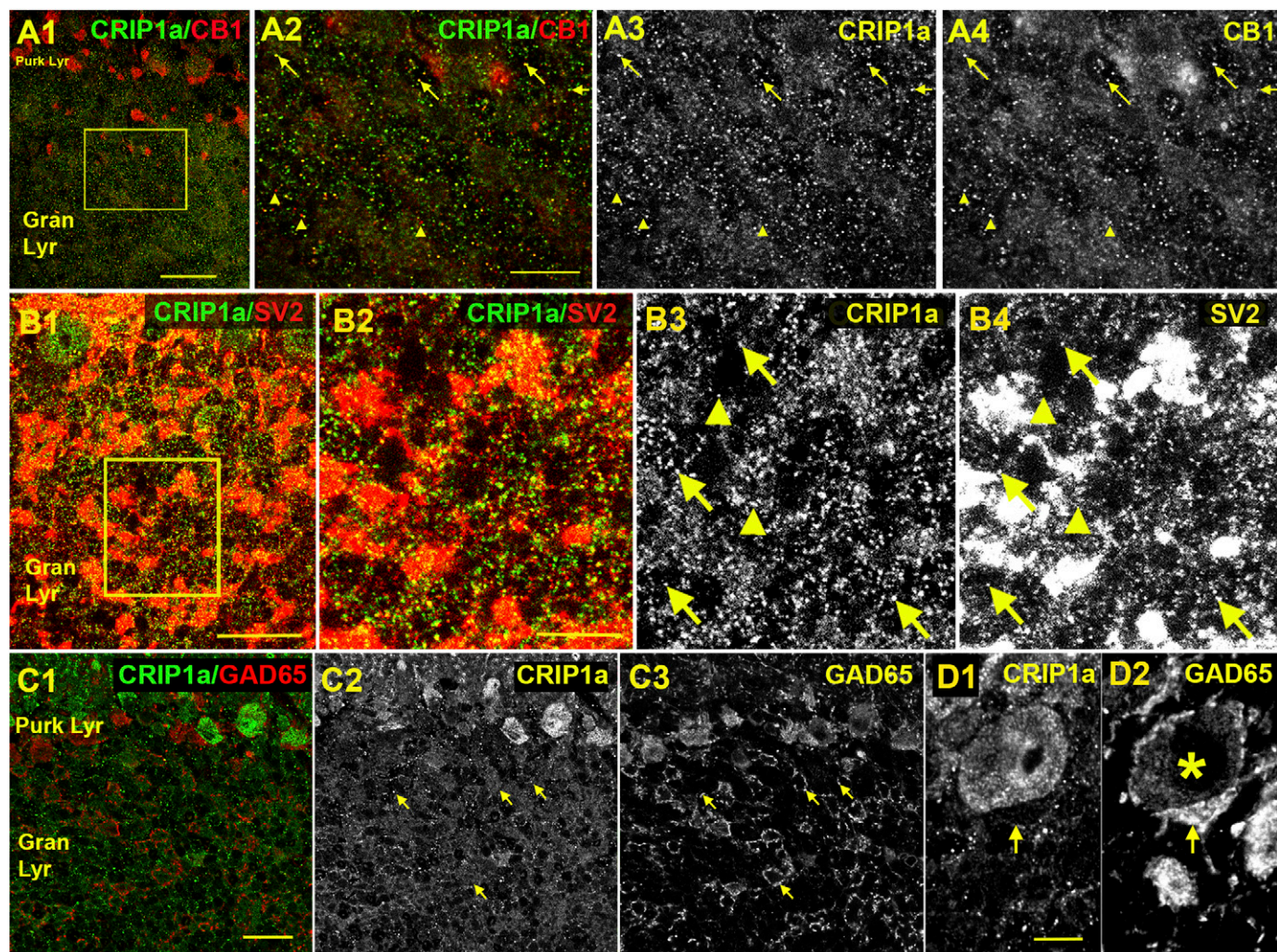
**Fig. 1.** Quantitative CRIP<sub>1a</sub> immunoblots and immunohistochemical localization of CRIP<sub>1a</sub> in rat cerebellum. CRIP<sub>1a</sub> was immunologically identified by (A and B) immunoblotting or (C) immunofluorescence staining. Quantitative immunoblotting was conducted by comparison of a standard curve of purified CRIP<sub>1a</sub> with CRIP<sub>1a</sub> immunoreactivity in membrane homogenates from (A) CB<sub>1</sub>-HEK cells with and without stable cotransfection of CRIP<sub>1a</sub> or (B) rat cerebellum. (C) Immunofluorescence staining of CRIP<sub>1a</sub> (green) and CB<sub>1</sub> receptor (red), or overlay (yellow) indicates areas of colocalization in rat cerebellum as determined by confocal microscopy. Note the characteristic scarcity of CB<sub>1</sub>R-ir in the granule cell layer (GCL) and colocalization of CB<sub>1</sub>R and CRIP<sub>1a</sub>-ir in the molecular layer (ML). Arrows indicate CB<sub>1</sub>R-ir in perisomatic basket cell axon terminals, which are devoid of CRIP<sub>1a</sub>-ir. PCL, Purkinje cell layer. Scale bar, 200  $\mu$ m.



**Fig. 2.** Immunohistochemical localization of CRIP<sub>1a</sub> in the cerebellum of GAD67-GFP transgenic mice. (A) Overview of CRIP<sub>1a</sub> staining versus GAD67-GFP in murine cerebellum shows a broad distribution in both the molecular (Mol) and granular (Gran) layers. (B) CRIP<sub>1a</sub> distribution near Purkinje cells. (C) CRIP<sub>1a</sub> protein in granular layer. (D) CRIP<sub>1a</sub> overlaps substantially with presynaptic marker SV2 in the molecular layer. In the adjacent image (E), CRIP<sub>1a</sub> also partially overlaps with GAD67-GFP neurons (arrows), including probable Purkinje cell processes. (F) Sample of CB<sub>1</sub>R colocalization with CRIP<sub>1a</sub> (arrows) in the molecular layer near Purkinje cells. Purkinje pinceau region is dense with CB<sub>1</sub> (arrowhead). Scale bars, (A) 100  $\mu$ m; (B) 25  $\mu$ m; (C) 30  $\mu$ m; and (D–F) 10  $\mu$ m.

pinceau region near Purkinje cells (e.g., arrowhead Fig. 2F) where the most intense CB<sub>1</sub>R expression is seen. Higher magnification images in the granule cell layer showed that CRIP<sub>1a</sub> is commonly colocalized with CB<sub>1</sub>Rs (Fig. 3A). Indeed most CRIP<sub>1a</sub> puncta overlapped with CB<sub>1</sub>R-ir, although there were some CB<sub>1</sub>R-positive puncta that were not positive for

CRIP<sub>1a</sub>. The diffuse CRIP<sub>1a</sub> staining appeared to correspond to mossy terminal staining as shown by overlap with the brighter SV2 staining (Fig. 3B). However, the most punctate CRIP<sub>1a</sub> staining, seen when mossy terminal staining was allowed to saturate (Fig. 3, B2–B4), also partially overlapped with SV2. CRIP<sub>1a</sub> staining in the granule cell layer did not colocalize with



**Fig. 3.** Colocalization of CRIP<sub>1a</sub> with CB<sub>1</sub>R, SV2, GAD65, and parvalbumin in the murine cerebellar granule cell layer. (A) Staining of CRIP<sub>1a</sub> (green) versus CB<sub>1</sub>R (red) shows numerous points of overlap in the granular layer of the murine cerebellum (arrows). But there are also clear cases where CB<sub>1</sub>R expression does not overlap with CRIP<sub>1a</sub> (arrowheads). Even punctate CRIP<sub>1a</sub> staining that appears to be green is often accompanied by CB<sub>1</sub>R staining (e.g., left arrow). Note larger CB<sub>1</sub>R-positive structures correspond to pinceau staining. (B) CRIP<sub>1a</sub> (green) is commonly associated with the presynaptic marker SV2 (red), both in the bright staining corresponding to mossy terminals but also, if the image is allowed to saturate as in this case, with a subset of isolated puncta (arrows). However nonoverlap also occurs (arrowheads). (C) CRIP<sub>1a</sub> (green) does not overlap with GAD65 (red) staining (arrows). (D) CRIP<sub>1a</sub> is absent in the pinceau region as identified by GAD65 staining (D1). Purkinje cell is marked by an asterisk. Scale bars, (A1) 35  $\mu$ m; (A2–A4) 15  $\mu$ m; (B1) 25  $\mu$ m; (B2–4) 10  $\mu$ m; (C) 20  $\mu$ m; (D) 5  $\mu$ m. Gran Lyr, granular layer; Purk Lyr, Purkinje layer.

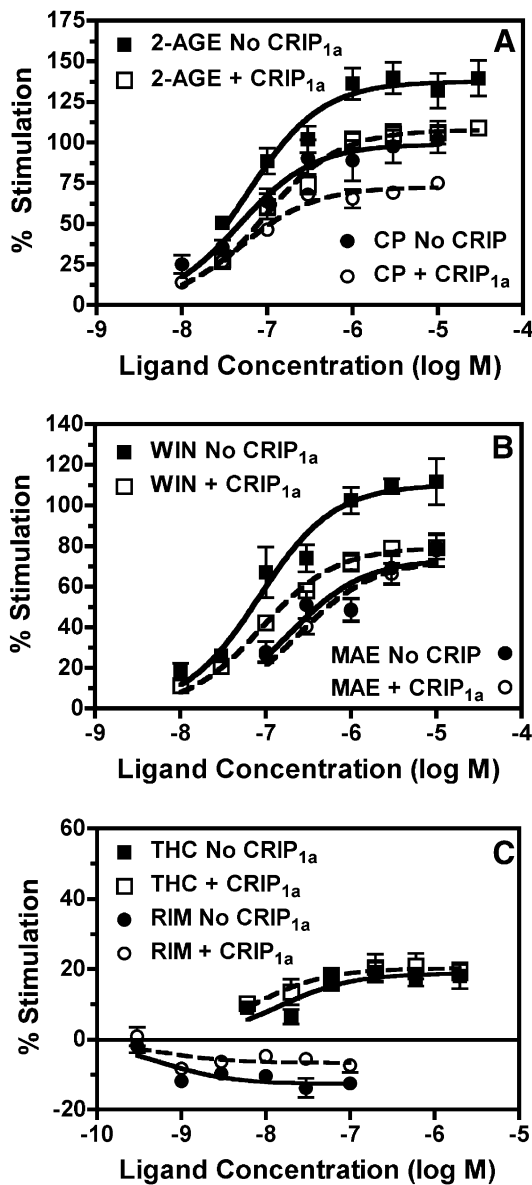
GAD65-ir (Fig. 3C) or GAD67-GFP (data not shown), suggesting that expression is restricted to excitatory cells in the granule layer (Meyer et al., 2002). It is notable that CRIP<sub>1a</sub> is not detected in the pinceau region of basket cell inputs to the Purkinje neurons (Fig. 3D), an area that is associated with strong CB<sub>1</sub>R expression (e.g., Fig. 2F). These results indicate widespread colocalization of CRIP<sub>1a</sub> with CB<sub>1</sub>R in multiple cellular elements of the cerebellum, although CRIP<sub>1a</sub> and CB<sub>1</sub>R expression did not completely overlap in all cerebellar subregions examined.

**Effects of CRIP<sub>1a</sub> on CB<sub>1</sub>R-Mediated G-Protein Activation in Stably Transfected HEK-293 Cells.** To determine the effects of CRIP<sub>1a</sub> on CB<sub>1</sub>R mediated G-protein activity, basal and ligand-modulated [<sup>35</sup>S]GTP $\gamma$ S binding was conducted in CB<sub>1</sub>-HEK ( $\pm$ CRIP<sub>1a</sub>) cells (Fig. 4). Concentration-effect curves for a variety of cannabinoid ligands were examined, including the classic phytocannabinoid THC, and its synthetic analogs HU210 and levonantradol; the aminoalkylindole WIN55,212-2;

the bicyclic cannabinoid CP55,940; the eicosanoids noladin ether [2-arachidonoylglycerol ether (2-AGE)], a stable analog of 2-AG that is also a putative endocannabinoid, and methanandamide (MAEA), a stable analog of anandamide; and the diarylpyrazole inverse agonist rimonabant, also known as SR141716A. Nonlinear regression fitting of the concentration-effect curves revealed maximal stimulation ( $E_{max}$ ) and EC<sub>50</sub> values for each ligand (Table 2). Results in CB<sub>1</sub>-HEK cells showed that noladin ether, WIN55,212-2 and HU210 appeared to act as full agonists (Fig. 4; Table 2). CP55,212-2 acted as a high efficacy partial agonist relative to 2-AGE, and MAEA and levonantradol were moderate efficacy partial agonists. THC acted as a low efficacy partial agonist and rimonabant acted as an inverse agonist (Fig. 4; Table 2).

In agreement with our previous findings (Niehaus et al., 2007), stable CRIP<sub>1a</sub> expression reduced the apparent inverse agonism of rimonabant compared with CB<sub>1</sub>-HEK cells without CRIP<sub>1a</sub> transfection (Fig. 4), as indicated by a main effect of cell





**Fig. 4.** Effects of CRIP<sub>1a</sub> overexpression on concentration-effect curves of ligand-modulated  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding in CB<sub>1</sub>-HEK cell membranes. Membranes from CB<sub>1</sub>-HEK cells with (open symbols) and without (closed symbols) stable cotransfection of CRIP<sub>1a</sub> were incubated (as described in *Materials and Methods*) with 100 mM NaCl, 10  $\mu\text{M}$  GDP, 0.1 nM  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ , and varying concentrations of the indicated ligands: (A) noladin ether (2-AGE) and CP55,940 (CP); (B) WIN55,212-2 (WIN) or methanandamide (MAE); or (C)  $\Delta^9$ -tetrahydrocannabinol or rimonabant (RIM). Data are mean percentage stimulation  $\pm$  S.E.M. ( $n = 3$ –6). No CRIP<sub>1a</sub>, no transfection of CRIP<sub>1a</sub>; CRIP<sub>1a</sub>, stable transfection of CRIP<sub>1a</sub>.

line in two-way ANOVA (cell line  $\times$  ligand concentration) of the concentration-effect curves (Table 2). This reduction in inverse agonism was attributable to a lesser maximal inhibition of basal G-protein activation by rimonabant in CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> than in CB<sub>1</sub>-HEK cells (Table 2). However,  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding measured in the absence of cannabinoid ligand (basal) did not differ between cell types. Basal binding in CB<sub>1</sub>-HEK cells was  $89.8 \pm 6.1$  fmol/mg and in CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells was  $86.4 \pm 5.5$  fmol/mg. Stable expression of CRIP<sub>1a</sub> also reduced stimulation of G-protein activation by the high efficacy agonists 2-AGE, WIN55,212-2, HU210, and CP55,940 (Fig. 4; Table 2),

as indicated by an effect of cell line in two-way ANOVA. This decrease in agonist-stimulated activity was attributable to a reduction in maximal stimulation ( $E_{\text{max}}$ ) in CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> relative to CB<sub>1</sub>-HEK cells, without any significant differences in  $\text{EC}_{50}$  values between the cell lines (Table 2).

Interestingly, concentration-effect curves for G-protein activation by the partial agonists MAEA, levonantradol, and THC were unaffected by CRIP<sub>1a</sub> overexpression (Fig. 4; Table 2). There was no significant effect of cell line in the presence of any of these partial agonists, and neither the  $E_{\text{max}}$  or  $\text{EC}_{50}$  values of these ligands differed between CB<sub>1</sub>-HEK and CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells. Because of the differential effect of CRIP<sub>1a</sub> on G-protein activation by ligands of different intrinsic efficacies, the relative efficacy relationship among some ligands differed between cell types. In CB<sub>1</sub>-HEK cells the order of descending relative efficacy, on the basis of  $E_{\text{max}}$  values, was 2-AGE  $\geq$  HU210 = WIN55,212-2  $\geq$  CP55,940  $\geq$  MAEA = levonantradol > THC >> rimonabant, whereas in CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells it was 2-AGE > HU210 > WIN55,212-2 = CP55,940 = MAEA = levonantradol > THC >> rimonabant.

The results described above indicate that stable CRIP<sub>1a</sub> expression in CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells did not inhibit basal  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding or  $[^3\text{H}]\text{SR141716A}$  binding. However, CRIP<sub>1a</sub> inhibited the inverse agonist effects of rimonabant under conditions of high  $[\text{Na}^+]$ , which reduces constitutive GPCR activity (Seifert and Wenzel-Seifert, 2002). We therefore determined whether CRIP<sub>1a</sub> inhibits constitutive receptor activity that is unrestricted by sodium. Results showed that 100 mM NaCl reduced basal  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding in both CB<sub>1</sub>-HEK and CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells by >50% relative to the absence of  $\text{Na}^+$  (Fig. 5A), as confirmed by two-way ANOVA (effect of  $\text{Na}^+$ ,  $P < 0.0001$ ). To distinguish receptor-mediated from receptor-independent G-protein activity, cells were pretreated with or without pertussis toxin (PTX), which uncouples G<sub>i/o</sub>-proteins from receptor-stimulated guanine nucleotide exchange (Sunyer et al., 1989).  $\text{Na}^+$  also inhibited basal  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding by 38% in both cell lines after treatment with PTX (effect of sodium,  $P < 0.0001$ ), but the effect was diminished relative to untreated cells (Fig. 5B). Importantly, CRIP<sub>1a</sub> inhibited basal  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding in untreated cells (effect of CRIP<sub>1a</sub>,  $P = 0.009$ ), but not in cells pretreated with PTX (no effect of CRIP<sub>1a</sub>,  $P = 0.496$ ). The inhibitory effect of CRIP<sub>1a</sub> on basal  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding was significant only in untreated cells in the absence of  $\text{Na}^+$ , as confirmed by Bonferroni posthoc analysis. These results indicate that CRIP<sub>1a</sub> inhibits spontaneous receptor-mediated G-protein activity, particularly in the absence of sodium, but does not affect receptor-independent  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding.

To determine the effects of  $\text{Na}^+$  on CB<sub>1</sub>R ligand-modulated G-protein activity, net  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding was determined in the presence of a maximally effective concentration of WIN55,212-2 or rimonabant with and without 100 mM NaCl in CB<sub>1</sub>-HEK and CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells.  $\text{Na}^+$  enhanced net stimulated  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding by WIN55,212-2 (effect of  $\text{Na}^+$ ,  $P < 0.0001$ ), and there was a significant interaction between  $\text{Na}^+$  and CRIP<sub>1a</sub> ( $P = 0.0346$ ), as indicated by two-way ANOVA (Fig. 6A). In the absence of  $\text{Na}^+$ , WIN55,212-2 had no effect on  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding. In contrast, WIN55,212-2 stimulated  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding in the presence of 100 mM  $\text{Na}^+$  ( $P < 0.05$  by Bonferroni posthoc test). The opposite effect of  $\text{Na}^+$  was seen with rimonabant (Fig. 6B). There was a significant effect of both  $\text{Na}^+$  ( $P = 0.0024$ ) and CRIP<sub>1a</sub> ( $P = 0.0466$ ) on net

TABLE 2

$E_{\max}$  and  $EC_{50}$  values of ligand-modulated [ $^{35}$ S]GTP $\gamma$ S binding in CB $_1$ -HEK and CB $_1$ -HEK-CRIP $_{1a}$  cells

Varying concentrations of the indicated ligands were incubated with membranes prepared from the indicated cell lines in the presence of 10  $\mu$ M GDP and 0.1 nM [ $^{35}$ S]GTP $\gamma$ S, as described in *Materials and Methods*.  $E_{\max}$  and  $EC_{50}$  values were derived from nonlinear regression analysis of ligand concentration-effect curves. Data are mean values  $\pm$  S.E.M. ( $n = 3-6$ ). The  $P$  values in the rightmost column denote significance of the effect of CRIP $_{1a}$  overexpression, derived from two-way ANOVA (ligand concentration  $\times$  cell line) of the concentration effect curves. Values  $<0.05$  are considered significant. Significance of  $E_{\max}$  and  $EC_{50}$  values between cell lines were determined by Student's  $t$  test.

Ligand	CB $_1$ -HEK		CB $_1$ -HEK-CRIP $_{1a}$		$P$ value
	$E_{\max}$	$EC_{50}$	$E_{\max}$	$EC_{50}$	
	% Stim	nM	% Stim	nM	
2-AGE	137.7 $\pm$ 9.7 <sup>a</sup>	59.9 $\pm$ 3.8	108.2 $\pm$ 3.7 <sup>a,*</sup>	104 $\pm$ 16	<0.0001
HU210	114.6 $\pm$ 5.9 <sup>a,b</sup>	0.04 $\pm$ 0.01	96.0 $\pm$ 6.1 <sup>b,§</sup>	0.06 $\pm$ 0.01	<0.0001
WIN55,212-2	112.7 $\pm$ 8.8 <sup>a,b,c</sup>	110 $\pm$ 41	79.5 $\pm$ 1.7 <sup>c,*</sup>	90.6 $\pm$ 6.2	<0.0001
CP55,940	100.0 $\pm$ 8.7 <sup>b,c</sup>	48.8 $\pm$ 2.2	72.0 $\pm$ 2.7 <sup>c,*</sup>	51.4 $\pm$ 6.3	<0.0001
MAEA	73.8 $\pm$ 6.1 <sup>c</sup>	202 $\pm$ 37	73.0 $\pm$ 6.0 <sup>c</sup>	255 $\pm$ 40	0.4928
Levonantradol	73.7 $\pm$ 11.3 <sup>c</sup>	34.2 $\pm$ 8.6	72.8 $\pm$ 0.5 <sup>c</sup>	19.0 $\pm$ 5.2	0.2360
THC	19.6 $\pm$ 2.7 <sup>d</sup>	11.5 $\pm$ 2.4	20.4 $\pm$ 3.1 <sup>d</sup>	8.1 $\pm$ 1.2	0.1740
Rimonabant	-12.9 $\pm$ 1.5	1.0 $\pm$ 0.4	-6.5 $\pm$ 1.5 <sup>*</sup>	0.5 $\pm$ 0.1	0.0002

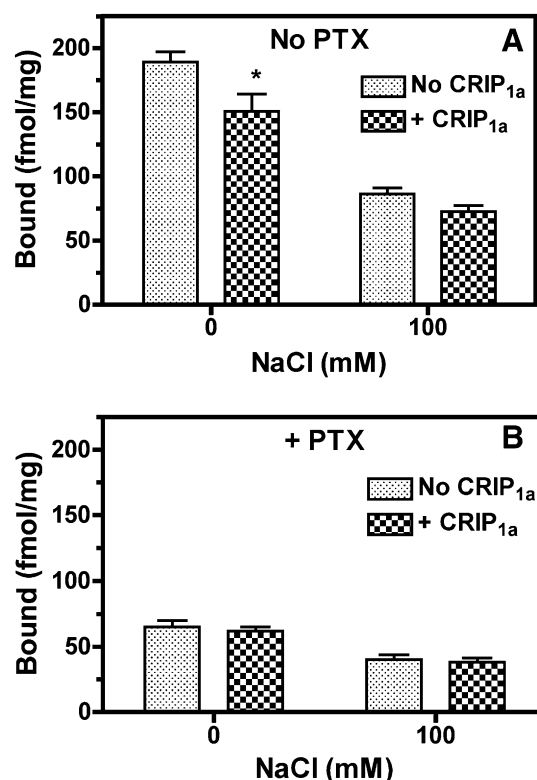
\* $P < 0.05$  different from CB $_1$ -HEK cells. § $P = 0.05$  different from CB $_1$ -HEK cells. <sup>a-d</sup>Significance of  $E_{\max}$  and  $EC_{50}$  values between cell lines were determined by Student's  $t$  test. Significant differences between ligand  $E_{\max}$  values within each cell line are denoted as follows: ligands without any similar letter designations are  $P < 0.05$  different from each other as determined by one-way ANOVA with posthoc Newman-Keuls test.

rimonabant-inhibited [ $^{35}$ S]GTP $\gamma$ S binding by two-way ANOVA. However, posthoc analysis revealed that rimonabant only significantly inhibited [ $^{35}$ S]GTP $\gamma$ S binding in the absence of Na<sup>+</sup> ( $P < 0.05$  by Bonferroni test). These results indicate that CRIP $_{1a}$  maximally attenuates agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in the presence of high [Na<sup>+</sup>] and maximally attenuates inverse agonist-inhibited [ $^{35}$ S]GTP $\gamma$ S binding in the absence of Na<sup>+</sup>, suggesting that CRIP $_{1a}$  attenuates both agonist-stimulated and constitutive CB $_1$ R-mediated G-protein activation in a manner consistent with the effects of Na<sup>+</sup> on basal receptor activity seen in Fig. 5.

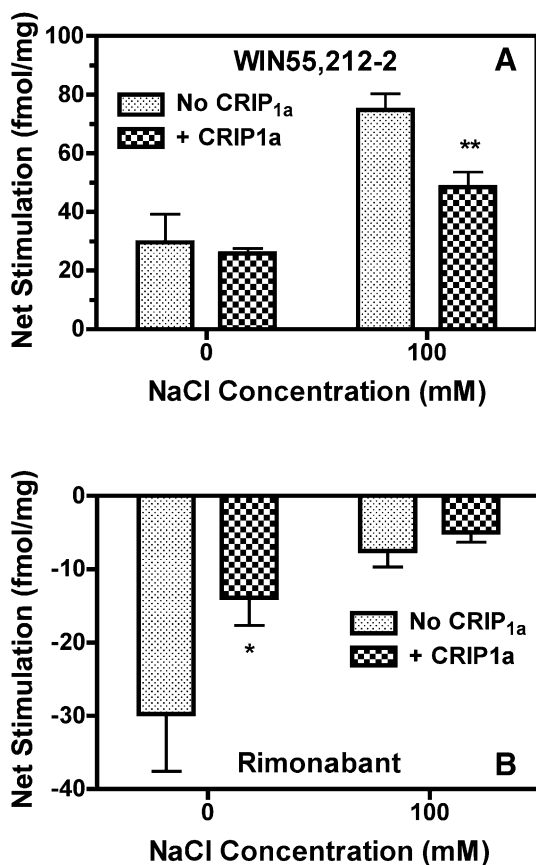
A potential confounding factor in the interpretation of the effects of CRIP $_{1a}$  on basal CB $_1$ R-mediated G-protein activity is the possible presence of endocannabinoids in the membrane preparation. To determine whether endocannabinoids could have contributed to basal G-protein activity, mass spectrometric analysis of CB $_1$ -HEK and CB $_1$ -HEK-CRIP $_{1a}$  cells and isolated cell membranes was performed to quantify the two major endocannabinoids, 2-AG and AEA. However, endocannabinoid levels were below the limit of detection for both 2-AG ( $>0.063$  pmol) and AEA ( $>0.039$  nmol) relative to deuterated standards, in extracts of both intact cells and isolated membranes, as determined in analysis of three independent samples. These results indicate that endocannabinoids are unlikely to have contributed to apparent basal CB $_1$ R-mediated G-protein activity in either cell line.

**Effect of CRIP $_{1a}$  on CB $_1$ R Desensitization and Down-regulation in Stably Transfected HEK-293 Cells.** To determine whether CRIP $_{1a}$  affected the regulation of CB $_1$ R function by prolonged agonist occupancy, CB $_1$ -HEK cells with and without stable CRIP $_{1a}$  cotransfection were pretreated with WIN55,212-2 (10  $\mu$ M), THC (6  $\mu$ M), or vehicle for 4 hours, followed by MAEA-stimulated [ $^{35}$ S]GTP $\gamma$ S binding to assess CB $_1$ R function. MAEA was used to assess CB $_1$ R activation after prolonged ligand pretreatment because acute stimulation of [ $^{35}$ S]GTP $\gamma$ S binding by this ligand was unaffected by CRIP $_{1a}$  (Fig. 2B; Table 2). Pretreatment of the cells with WIN55,212-2 or THC decreased CB $_1$ R-mediated G-protein activation in membranes prepared from either cell line (Fig. 7). Significantly lower MAEA  $E_{\max}$  values were seen in WIN55,212-2-pretreated

compared with vehicle-pretreated cells with or without CRIP $_{1a}$  cotransfection (Table 3). In contrast, THC pretreatment did not affect MAEA  $E_{\max}$  values in either cell line. However, pretreatment with either drug significantly increased MAEA



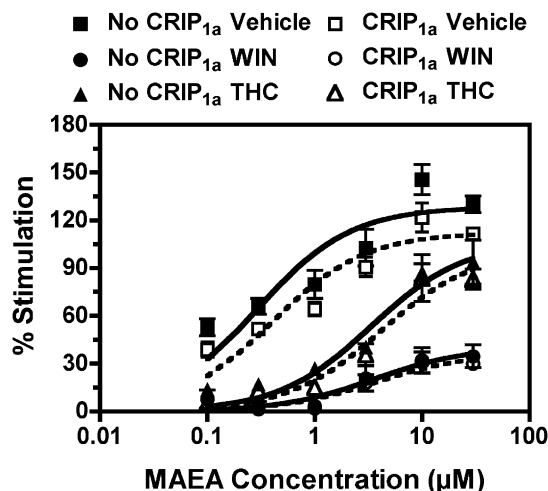
**Fig. 5.** CRIP $_{1a}$  overexpression in CB $_1$ -HEK cells suppressed basal [ $^{35}$ S]GTP $\gamma$ S binding in the absence of sodium in a PTX-sensitive manner. Membranes from CB $_1$ -HEK cells with and without stable cotransfection of CRIP $_{1a}$  were pretreated for 24 hours with and without 50 ng/ml PTX and were then incubated with 10  $\mu$ M GDP and 0.1 nM [ $^{35}$ S]GTP $\gamma$ S in the presence and absence of 100 mM NaCl (as described in *Materials and Methods*). Data are mean fmol/mg bound  $\pm$  S.E.M. ( $n = 4$ ). \* $P < 0.05$  different from cells without CRIP $_{1a}$  cotransfection under the corresponding conditions, as determined by two-way ANOVA with Bonferroni posthoc test. No CRIP $_{1a}$ , no transfection of CRIP $_{1a}$ ; CRIP $_{1a}$ , stable transfection of CRIP $_{1a}$ .



**Fig. 6.** CRIP<sub>1a</sub> overexpression in CB<sub>1</sub>-HEK cells affects net ligand-modulated [<sup>35</sup>S]GTP $\gamma$ S binding in a sodium-dependent manner. Membranes from CB<sub>1</sub>-HEK cells with and without stable cotransfection of CRIP<sub>1a</sub> were incubated with 10  $\mu$ M GDP and 0.1 nM [<sup>35</sup>S]GTP $\gamma$ S in the presence and absence of 100 mM NaCl (as described in *Materials and Methods*). Data are mean fmol/mg bound  $\pm$  S.E.M. ( $n = 4-5$ ). \* $P < 0.05$ , \*\* $P < 0.01$  different from cells without CRIP<sub>1a</sub> cotransfection under the corresponding conditions, as determined by two-way ANOVA with Bonferroni posthoc test. No CRIP<sub>1a</sub>; no transfection of CRIP<sub>1a</sub>; CRIP<sub>1a</sub>, stable transfection of CRIP<sub>1a</sub>.

EC<sub>50</sub> values (Table 3). Thus, pretreatment with either WIN55,212-2 or THC apparently desensitized MAEA-stimulated G-protein activity in both cell lines. However, there were no apparent differences in the level of desensitization produced by either ligand between CB<sub>1</sub>-HEK cells with and without stable CRIP<sub>1a</sub> cotransfection. Indeed, two-way ANOVA of  $E_{max}$  values indicated a significant effect of drug pretreatment ( $P < 0.0001$ ), but there was no effect of CRIP<sub>1a</sub> ( $P = 0.306$ ). Likewise, two-way ANOVA of EC<sub>50</sub> values revealed a significant effect of drug pretreatment ( $P = 0.0036$ ), but there was no effect of CRIP<sub>1a</sub> ( $P = 0.848$ ). Subsequent one-way ANOVA with posthoc Newman-Keuls multiple comparison test revealed that neither  $E_{max}$  nor EC<sub>50</sub> values of MAEA differed significantly between CB<sub>1</sub>-HEK cells with and without stable CRIP<sub>1a</sub> cotransfection after WIN55,212-2 or THC pretreatment. These results indicate that CRIP<sub>1a</sub> did not affect cannabinoid-induced CB<sub>1</sub>R desensitization under these conditions.

Basal [<sup>35</sup>S]GTP $\gamma$ S binding was not significantly affected by ligand pretreatment in either cell line (Supplemental Fig. 4). Basal [<sup>35</sup>S]GTP $\gamma$ S binding in vehicle-pretreated cells was  $48.9 \pm 9.2$  and  $43.9 \pm 4.2$  fmol/mg in cells without and with CRIP<sub>1a</sub> coexpression, respectively. There was no significant effect of



**Fig. 7.** CRIP<sub>1a</sub> does not affect ligand-induced desensitization of CB<sub>1</sub>R-mediated G-protein activation in CB<sub>1</sub>-HEK cells. Cells were pretreated for 4 hours with 10  $\mu$ M WIN55,212-2 (WIN), 6  $\mu$ M THC, or vehicle prior to harvesting and preparation of membranes. Varying concentrations of MAEA were incubated with membranes prepared from the indicated cell lines in the presence of 100 mM NaCl, 10  $\mu$ M GDP, and 0.1 nM [<sup>35</sup>S]GTP $\gamma$ S, as described in *Materials and Methods*. Data are mean percentage stimulation  $\pm$  S.E.M. ( $n = 4$ ). No CRIP<sub>1a</sub>; no transfection of CRIP<sub>1a</sub>; CRIP<sub>1a</sub>, stable transfection of CRIP<sub>1a</sub>.

pretreatment ( $P = 0.584$ ) or CRIP<sub>1a</sub> expression ( $P = 0.547$ ) with regard to basal [<sup>35</sup>S]GTP $\gamma$ S binding, according to two-way ANOVA. Likewise, one-way ANOVA revealed no significant effect of pretreatment in either cell line. These results indicate that the pretreatment ligand was sufficiently removed prior to assay, because residual agonist that might have remained in the membrane preparation from pretreated cells would be predicted to elevate the apparent “basal” level of [<sup>35</sup>S]GTP $\gamma$ S binding.

In contrast, CRIP<sub>1a</sub> overexpression attenuated cannabinoid ligand-induced CB<sub>1</sub>R downregulation, as determined using the identical pretreatment protocol that was used to examine effects on G-protein activation. Two-way ANOVA of [<sup>3</sup>H]SR141716A  $B_{max}$  values revealed significant effects of ligand pretreatment ( $P = 0.0001$ ) but no effect of CRIP<sub>1a</sub> expression ( $P = 0.199$ ). However, there was a trend toward an interaction between these two factors ( $P = 0.06$ ). With regard to [<sup>3</sup>H]SR141716A  $K_D$  values, there was no effect of ligand pretreatment ( $P = 0.812$ ) or CRIP<sub>1a</sub> expression ( $P = 0.345$ ), nor was there an interaction ( $P = 0.736$ ). In CB<sub>1</sub>-HEK cells, pretreatment with either 10  $\mu$ M WIN55,212-2 or 6  $\mu$ M THC decreased the  $B_{max}$  value of [<sup>3</sup>H]SR141716A binding by 53 and 74%, respectively (Table 3), as determined by one-way ANOVA with posthoc Dunnett’s test. However, analysis of  $B_{max}$  values in CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells showed no significant effect of ligand pretreatment by one-way ANOVA ( $P = 0.274$ ). Likewise, one-way ANOVA revealed no significant effects of pretreatment on  $K_D$  values in either CB<sub>1</sub>-HEK ( $P = 0.734$ ) or CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> ( $P = 0.798$ ) cells. These results indicate that pretreatment with either WIN55,212-2 or THC significantly downregulated CB<sub>1</sub>Rs in CB<sub>1</sub>-HEK cells, but not in CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells. Moreover, the lack of effect of ligand pretreatment on [<sup>3</sup>H]SR141716A  $K_D$  values in either cell line further indicates that the effects of these pretreatments on CB<sub>1</sub>R levels or activation of G-proteins were not attributable to insufficient removal of the pretreatment ligand prior to assay.

TABLE 3

Curve-fit values of MAEA-stimulated [<sup>35</sup>S]GTPγS and [<sup>3</sup>H]SR141716A binding in CB<sub>1</sub>-HEK and CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells pretreated with vehicle, WIN55,212-2, or THC

Cells were pretreated for 4 hours with 10 μM WIN55,212-2, 6 μM THC, or vehicle. Varying concentrations of MAEA were incubated with membranes prepared from the indicated cell lines in the presence of 10 μM GDP and 0.1 nM [<sup>35</sup>S]GTPγS, as described in *Materials and Methods*. Varying concentrations of [<sup>3</sup>H]SR141716A were incubated with membranes prepared from the indicated cell lines, as described in *Materials and Methods*. *E*<sub>max</sub>, EC<sub>50</sub>, *B*<sub>max</sub>, and *K*<sub>D</sub> values were derived from nonlinear regression analysis of the concentration-effect or saturation binding curves. Data are mean values ± S.E.M. (*n* = 4–5).

	Cell Line Pretreatment		
	Vehicle	WIN55,212-2	THC
MAEA-stimulated [ <sup>35</sup> S]GTPγS binding			
CB <sub>1</sub> -HEK			
<i>E</i> <sub>max</sub> (% Stim)	128.5 ± 8.0	42.7 ± 6.9**	100.5 ± 8.5
EC <sub>50</sub> (nM)	308 ± 71	5412 ± 1870 <sup>§</sup>	4609 ± 2137 <sup>§</sup>
CB <sub>1</sub> -HEK-CRIP <sub>1a</sub>			
<i>E</i> <sub>max</sub> (% Stim)	112.6 ± 5.1	36.9 ± 4.5**	104.3 ± 7.3
EC <sub>50</sub> (nM)	423 ± 82	4112 ± 633 <sup>§§</sup>	5175 ± 1316 <sup>§§</sup>
[ <sup>3</sup> H]SR141716A binding			
CB <sub>1</sub> -HEK			
<i>B</i> <sub>max</sub> (pmol/mg)	1.19 ± 0.11	0.56 ± 0.10**	0.31 ± 0.04**
<i>K</i> <sub>D</sub> (nM)	1.78 ± 0.23	1.37 ± 0.34	1.78 ± 0.59
CB <sub>1</sub> -HEK-CRIP <sub>1a</sub>			
<i>B</i> <sub>max</sub> (pmol/mg)	0.97 ± 0.15	0.86 ± 0.14	0.62 ± 0.12
<i>K</i> <sub>D</sub> (nM)	1.72 ± 0.46	2.18 ± 0.69	2.40 ± 1.07

\*\**P* < 0.01, different from vehicle-treated cells of the same type, as determined by one-way ANOVA with posthoc Dunnett's test; <sup>§</sup>*P* = 0.05, <sup>§§</sup>*P* < 0.01 different from vehicle-treated cells of the same type, as determined by one-way ANOVA of square root transformed data with posthoc Dunnett's test.

**Effects of CRIP<sub>1a</sub> on CB<sub>1</sub>R-Mediated G-Protein Activation in Stably Transfected N18TG2 Cells.** Results in CB<sub>1</sub>-HEK cells with and without stable cotransfection of CRIP<sub>1a</sub> indicated that CRIP<sub>1a</sub> negatively modulates constitutive and high efficacy agonist-stimulated G-protein activation by CB<sub>1</sub>Rs. To determine whether similar effects could be demonstrated in a neural cell type, mouse neuroblastoma N18TG2 cells, which endogenously express CB<sub>1</sub>Rs, were stably transfected with CRIP<sub>1a</sub>. In addition, because N18TG2 cells endogenously express CRIP<sub>1a</sub> at moderate levels that are in excess of CB<sub>1</sub>R levels (Supplemental Fig. 5, Table 4), cell lines with stable transfection of siRNA against CRIP<sub>1a</sub> were also generated. Two cloned cell lines (OX1 and OX5) were isolated that stably overexpressed CRIP<sub>1a</sub> mRNA without any alteration in CB<sub>1</sub>R mRNA (Supplemental Fig. 5B). CRIP<sub>1a</sub> clones expressed 8:1 and 7:1 (CRIP<sub>1a</sub>/CB<sub>1</sub>R) cDNA ratios, respectively, as compared with a 1:7 ratio in untransfected N18TG2 cells (determined by real-time polymerase chain reaction using eno2 as a standard; Supplemental Fig. 5A). Comparative immunoblots also indicated greater relative expression of CRIP<sub>1a</sub> protein in CRIP<sub>1a</sub>-overexpressing clones compared with untransfected N18TG2 cells (Supplemental Fig. 5C). Likewise, two clones (KD2C and KD2F) were isolated that exhibited siRNA-mediated knockdown of CRIP<sub>1a</sub> mRNA relative to untransfected N18TG2 cells, whereas cells transfected with empty siRNA vector did not exhibit CRIP<sub>1a</sub> knockdown (Supplemental Fig. 5A). Immunoblot analysis showed that CRIP<sub>1a</sub> protein levels were reduced by 50–60% in siRNA knockdown clones relative to untransfected or vector control-transfected N18TG2 cells (Supplemental Fig. 5C), but CB<sub>1</sub>R protein levels did not differ between any of these cell models (Supplemental Fig. 5D).

To determine the precise level of membrane-delimited CRIP<sub>1a</sub> protein expression in CRIP<sub>1a</sub>-OX N18TG2 clones for comparison with results in CRIP<sub>1a</sub>-overexpressing CB<sub>1</sub>-HEK cells, quantitative immunoblots using purified CRIP<sub>1a</sub> standards were then

conducted in isolated membranes prepared from the two CRIP<sub>1a</sub>-transfected clones and untransfected N18TG2 cells. Untransfected N18TG2 cells expressed 0.56 ± 0.06 pmol CRIP<sub>1a</sub> per mg of membrane protein, whereas clones OX1 and OX5 expressed 1.35 ± 0.16 pmol/mg and 1.28 ± 0.09 pmol/mg, respectively. The results of CRIP<sub>1a</sub> quantitative immunoblots were then compared with *B*<sub>max</sub> values derived from cannabinoid radioligand binding assays in control and CRIP<sub>1a</sub>-overexpressing N18TG2 cell lines to determine the stoichiometric ratio of CRIP<sub>1a</sub>/CB<sub>1</sub> and the effect of CRIP<sub>1a</sub> overexpression on CB<sub>1</sub>R *B*<sub>max</sub> values. Owing to low CB<sub>1</sub>R expression levels in N18TG2 cells, [<sup>3</sup>H]CP55,940 was used as the radioligand because it yielded greater specific/nonspecific binding ratios than [<sup>3</sup>H]SR141716A. Quantitative immunoblot and [<sup>3</sup>H]CP55,940 binding analysis indicated that CRIP<sub>1a</sub>-overexpressing N18TG2 clones have approximately a 2.3- to 2.5-fold increase in both CRIP<sub>1a</sub> protein expression and the CRIP<sub>1a</sub>/CB<sub>1</sub> expression ratio, in comparison with untransfected N18TG2 cells (Table 4).

In line with findings from stable CB<sub>1</sub>-HEK-CRIP<sub>1a</sub> cells, CRIP<sub>1a</sub> overexpression (clones OX1 and OX5) or knockdown (clone KD2C) did not alter the *B*<sub>max</sub> values of [<sup>3</sup>H]CP55,940 binding (Table 4), as determined by one-way ANOVA (*P* = 0.899). Likewise, [<sup>3</sup>H]CP55,940 *K*<sub>D</sub> values were not significantly affected by CRIP<sub>1a</sub> overexpression or knockdown (*P* = 0.145 by one-way ANOVA; Table 4). These results demonstrate altered expression of CRIP<sub>1a</sub> protein without altering CB<sub>1</sub>R density in both CRIP<sub>1a</sub>-overexpressing and knockdown cells compared with control N18TG2 cells. It is noteworthy to mention that the relative increase in CRIP<sub>1a</sub>/CB<sub>1</sub>R ratios in CRIP<sub>1a</sub>-overexpressing N18TG2 cells was less than that in the CB<sub>1</sub>-HEK cell models, where CRIP<sub>1a</sub>-overexpressing cells showed a 16-fold increase over control cells, as compared with ~2.5-fold increase in N18TG2-CRIP<sub>1a</sub> cells.

To determine whether CRIP<sub>1a</sub> overexpression in N18TG2 cells affected constitutive and agonist-stimulated G-protein activation by CB<sub>1</sub>Rs, ligand concentration-effect curves for

TABLE 4

Stoichiometry of CRIP<sub>1a</sub> and CB<sub>1</sub> receptor expression in N18TG2 and N18TG2-CRIP<sub>1a</sub> cells

Membranes prepared from the indicated cell line were incubated with varying concentrations of [<sup>3</sup>H]CP55,940, as described in *Materials and Methods*.  $B_{\max}$  and  $K_D$  values were derived from nonlinear regression analysis of the saturation binding curves. CRIP<sub>1a</sub> protein values were determined by quantitative immunoblot using purified CRIP<sub>1a</sub> as an internal standard, as described in *Materials and Methods*. Data are mean values  $\pm$  S.E.M. ( $n = 4-8$ ).

Tissue Source	CB <sub>1</sub> $B_{\max}$	CB <sub>1</sub> $K_D$	CRIP <sub>1a</sub>	Molar Ratio CRIP <sub>1a</sub> /CB <sub>1</sub>
	<i>pmol/mg</i>	<i>nM</i>	<i>pmol/mg</i>	
Control N18TG2	0.298 $\pm$ 0.039	2.35 $\pm$ 0.33	0.56 $\pm$ 0.07	1.87 $\pm$ 0.23
N18-CRIP <sub>1a</sub> -OX1	0.277 $\pm$ 0.033	3.02 $\pm$ 0.71	1.35 $\pm$ 0.16**	4.87 $\pm$ 0.59**
N18-CRIP <sub>1a</sub> -OX5	0.270 $\pm$ 0.050	4.22 $\pm$ 0.96	1.28 $\pm$ 0.09**	4.74 $\pm$ 0.34**
N18-CRIP <sub>1a</sub> -KD	0.320 $\pm$ 0.045	2.49 $\pm$ 0.38	N.D.	N.D.

\*\* $P < 0.01$  different from corresponding value in control N18TG2 cells by one-way ANOVA with posthoc Dunnett's test. N.D., not determined.

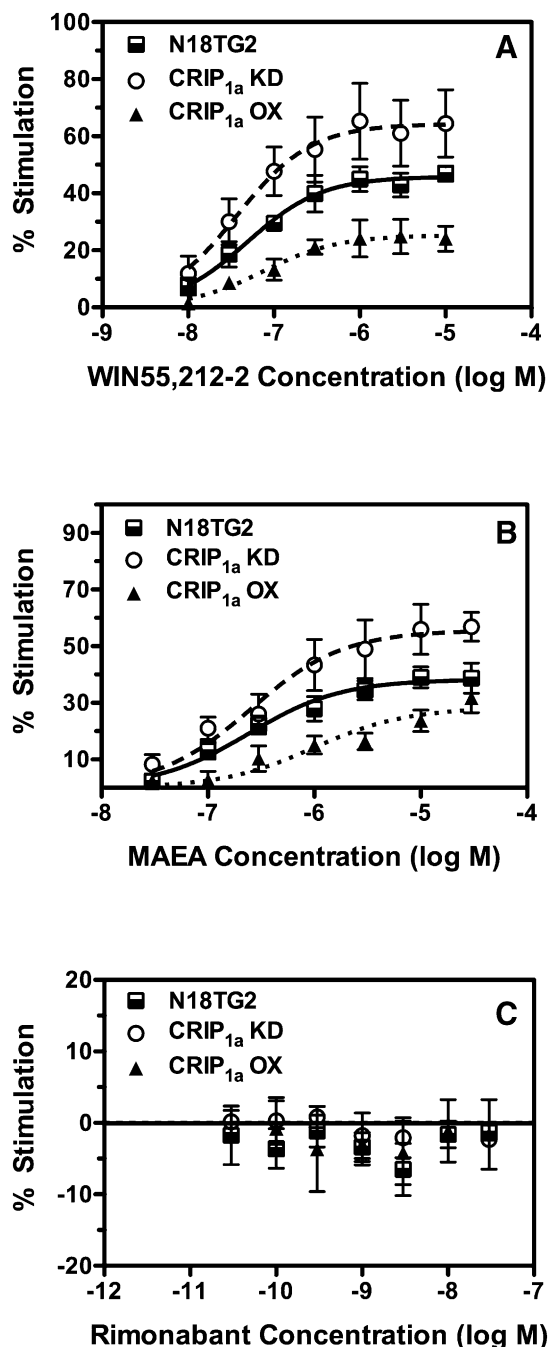
modulation of [<sup>35</sup>S]GTP $\gamma$ S binding were examined using WIN55,212-2, MAEA, and rimonabant in CRIP<sub>1a</sub> knockdown clone KD2C (N18-CRIP<sub>1a</sub>-KD), and overexpressing clone OX1 (N18-CRIP<sub>1a</sub>-OX) compared with untransfected N18TG2 cells. Basal [<sup>35</sup>S]GTP $\gamma$ S binding did not differ significantly between N18TG2 (68.1  $\pm$  6.0 fmol/mg), N18-CRIP<sub>1a</sub>-KD (66.6  $\pm$  16.6), and N18-CRIP<sub>1a</sub>-OX cells (70.5  $\pm$  5.9 fmol/mg). Results of ligand concentration-effect curves showed that CRIP<sub>1a</sub> overexpression decreased WIN55,212-2- and MAEA-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding relative to control N18TG2 cells (Fig. 8, A and B). There was a significant effect of CRIP<sub>1a</sub> knockdown on [<sup>35</sup>S]GTP $\gamma$ S binding stimulated by either WIN55,212-2 ( $P < 0.0001$ ) or MAEA ( $P < 0.0001$ ) in N18-CRIP<sub>1a</sub>-KD (clone KD2C) relative to control N18TG2 cells, as revealed by two-way ANOVA of the concentration-effect curves. Likewise, two-way ANOVA revealed a significant effect of CRIP<sub>1a</sub> overexpression on [<sup>35</sup>S]GTP $\gamma$ S binding stimulated by either WIN55,212-2 ( $P < 0.0001$ ) or MAEA ( $P < 0.0001$ ) in N18-CRIP<sub>1a</sub>-OX (clone OX1) relative to control N18TG2 cells. In contrast, rimonabant did not reliably inhibit basal [<sup>35</sup>S]GTP $\gamma$ S binding in a concentration-dependent manner in any of the three N18TG2 cell lines examined under these experimental conditions (Fig. 8C), in contrast to results obtained in the CB<sub>1</sub>-HEK cell models. Accordingly, there were no significant effects of either rimonabant concentration or CRIP<sub>1a</sub> expression levels in comparing the rimonabant concentration-effect curves among these three N18TG2 cell lines using two-way ANOVA. Similar results were obtained with all three ligands when comparing ligand-modulated [<sup>35</sup>S]GTP $\gamma$ S binding in the other CRIP<sub>1a</sub>-overexpressing (OX5) and knockdown (KD2F) clones to control N18TG2 cells (Supplemental Fig. 6 and Fig. 8).

Nonlinear regression analysis of the ligand concentration-effect curves showed that the  $E_{\max}$  values of both WIN55,212-2 and MAEA were significantly higher in N18-CRIP<sub>1a</sub>-KD relative to control N18TG2 cells (Table 5). Conversely, the WIN55,212-2  $E_{\max}$  value was lower in N18-CRIP<sub>1a</sub>-OX cells compared with control N18TG2 cells. However, the MAEA  $E_{\max}$  value did not differ significantly between N18-CRIP<sub>1a</sub>-OX cells and control N18TG2 cells, but both WIN55212-2 and MAEA  $E_{\max}$  values in N18-CRIP<sub>1a</sub>-OX cells were significantly lesser than in N18-CRIP<sub>1a</sub>-KD cells. In contrast, neither WIN55212-2 nor MAEA  $EC_{50}$  values were significantly altered by manipulation of CRIP<sub>1a</sub> expression levels in these N18TG2 cell lines. Moreover, because two-way ANOVA showed no significant effect of rimonabant concentration in these N18TG2 cell models, curve-fitting analysis was not performed with this ligand. These results indicate that CRIP<sub>1a</sub> knockdown enhances

whereas CRIP<sub>1a</sub> overexpression attenuates agonist-stimulated G-protein activation mediated by CB<sub>1</sub>Rs that are endogenously expressed in N18TG2 neuroblastoma cells.

Potential effects of CRIP<sub>1a</sub> on constitutive CB<sub>1</sub>R-mediated G-protein activation could not be determined in the N18TG2 cell models because rimonabant did not significantly inhibit basal [<sup>35</sup>S]GTP $\gamma$ S binding. It is possible, however, that endocannabinoids present in N18TG2 cells could have obscured the inhibitory effects of rimonabant on basal G-protein activity, perhaps by competing with rimonabant. To address this question, lipid fractions from N18TG2 and N18-CRIP<sub>1a</sub>-OX cells, or membranes isolated from each cell line, were analyzed by mass spectrometry to determine the content of 2-AG and AEA. Results showed detectable levels of 2-AG in both intact cells and membrane preparations, although levels on a per-cell basis were approximately 7.5-fold greater in intact cells than isolated membranes (Supplemental Fig. 7). Importantly, 2-AG levels did not differ between N18TG2 and N18-CRIP<sub>1a</sub>-OX cells in fractions prepared from either intact cells or isolated membranes. To determine whether the higher 2-AG levels in intact cells compared with membranes were attributable to greater lipase activity in cells, the cells were incubated in the presence and absence of the diacylglycerol lipase inhibitor tetrahydrolipstatin (orlistat). Results showed that orlistat treatment significantly decreased 2-AG levels in intact cell preparations but not in membranes (Supplemental Fig. 6). In intact cells, two-way ANOVA revealed a significant effect of orlistat ( $P = 0.032$ ) but not CRIP<sub>1a</sub> overexpression ( $P = 0.696$ ), and there was no significant interaction between the two factors ( $P = 0.768$ ). In isolated membranes, there was only a nonsignificant trend toward an effect of orlistat ( $P = 0.105$ ), and there was no effect of CRIP<sub>1a</sub> overexpression ( $P = 0.574$ ) and no interaction between the two factors ( $P = 0.860$ ). AEA levels were detectable in intact cells but were approximately 0.02% of the levels detected for 2-AG, or approximately 0.01 pmol/10<sup>7</sup> cells (data not shown). Two-way ANOVA revealed no effects of either CRIP<sub>1a</sub> overexpression ( $P = 0.540$ ) or orlistat ( $P = 0.452$ ), and there was no significant interaction (0.791). AEA levels in membrane preparations were below the limit of detection. These results suggest that differences in endocannabinoid levels were not responsible for the lack of inhibitory effects of rimonabant in membrane preparations from N18TG2 cells with and without overexpression of CRIP<sub>1a</sub>.

**Effects of CRIP<sub>1a</sub> on Endocannabinoid Signaling in Hippocampal Neuronal Cultures.** Results from both HEK-293 and N18TG2 cell models indicate that CRIP<sub>1a</sub> can negatively modulate agonist-stimulated CB<sub>1</sub>R activity at



**Fig. 8.** Effect of CRIP<sub>1a</sub> knockdown and overexpression on concentration-effect curves of ligand-modulated [<sup>35</sup>S]GTP $\gamma$ S binding in N18TG2 neuroblastoma cell membranes. Membranes from wild-type N18TG2 cells or N18TG2 cells with siRNA-mediated knockdown (CRIP<sub>1a</sub>-KD; clone 2C) or overexpression (CRIP<sub>1a</sub>-OX; clone 1) of CRIP<sub>1a</sub> were incubated as described in *Materials and Methods* with 100 mM NaCl, 20  $\mu$ M GDP, 0.1 nM [<sup>35</sup>S]GTP $\gamma$ S, and varying concentrations of WIN55,212-2, methanandamide, or rimonabant (RIM). Data are mean percentage stimulation  $\pm$  S.E.M. ( $n = 5-9$ ).

the level of G-protein activation. However, our previous results from isolated SCG neurons indicated that although CRIP<sub>1a</sub> overexpression attenuated constitutive CB<sub>1</sub>R-mediated Ca<sup>2+</sup> channel inhibition, it did not alter agonist-inhibited Ca<sup>2+</sup> channel activity (Niehaus et al., 2007). Therefore, the effects of CRIP<sub>1a</sub> overexpression on synaptic function of CB<sub>1</sub>Rs were examined in a more CNS-relevant model. Autaptic

hippocampal neurons express all the components of a functional cannabinoid signaling system, including presynaptic CB<sub>1</sub>Rs, depolarization-dependent production of endocannabinoids, probably 2-AG (Straiker and Mackie, 2005), and monoacylglycerol lipase, which hydrolyzes 2-AG and thereby controls the duration of cannabinoid signaling (Straiker et al., 2009). To assess whether CRIP<sub>1a</sub> can functionally interact with this endogenous cannabinoid signaling system, CRIP<sub>1a</sub> was overexpressed in autaptic neurons. The distribution of CRIP<sub>1a</sub> protein was determined in transfected neurons using an HA11 antibody against the HA tag on the CRIP<sub>1a</sub> protein, and results showed that CRIP<sub>1a</sub> was widely expressed throughout the transfected neuron (Fig. 9A). This widespread cellular localization was similar to that of endogenous CRIP<sub>1a</sub> (Fig. 9, B and D; compare with Figs. 1, 2, and 3). Although endogenously expressed CB<sub>1</sub>R appeared to be primarily limited to neuronal processes and putative autaptic terminals (Fig. 9, B and E), endogenous CRIP<sub>1a</sub> was widely colocalized in these cellular elements with CB<sub>1</sub>Rs (Fig. 9, B and F). These results suggest that CRIP<sub>1a</sub> is spatially positioned in a manner such that it could modulate CB<sub>1</sub>R function.

To determine whether CRIP<sub>1a</sub> overexpression altered the sensitivity of DSE induction in these autaptic hippocampal neurons, depolarization duration-response curves were obtained. Neurons were depolarized for progressively longer durations (50 milliseconds, 100 milliseconds, 300 milliseconds, 500 milliseconds, 1 second, 3 seconds, and 10 seconds) and the resulting CB<sub>1</sub>R-dependent inhibition was measured. Results showed that the depolarization duration-response curve in CRIP<sub>1a</sub>-overexpressing neurons differed substantially from that of control conditions, with a diminished inhibition at 1-, 3- and 10-second depolarizations (Fig. 10, A and B;  $P < 0.05$  by Bonferroni posthoc test after two-way ANOVA).

2-AG is a strong candidate to serve as the endocannabinoid-mediating DSE at autaptic hippocampal synapses (Straiker and Mackie, 2005, 2009; Jain et al., 2013). Therefore, the response in CRIP<sub>1a</sub>-transfected neurons to 2-AG was examined to determine whether sensitivity to exogenously added 2-AG was diminished to the same degree as was DSE. Figure 10C shows that inhibition of EPSCs by 2-AG (5  $\mu$ M) was substantially attenuated in CRIP<sub>1a</sub>-overexpressing neurons relative to controls [relative EPSC charge with 2-AG (5  $\mu$ M) treatment in wild-type neurons:  $0.34 \pm 0.03$ ,  $n = 4$ ; in CRIP<sub>1a</sub>-transfected neurons:  $0.82 \pm 0.06$ ,  $n = 7$ ;  $P < 0.05$  by unpaired  $t$  test]. We have previously reported that anandamide activates CB<sub>1</sub>Rs to inhibit neurotransmitter release in excitatory and inhibitory autaptic neurons (Straiker and Mackie, 2005, 2009). Thus, the hydrolysis-resistant analog MAEA was tested under control and CRIP<sub>1a</sub>-transfected conditions. As with 2-AG, CRIP<sub>1a</sub> overexpression also diminished MAEA signaling [Fig. 10C; relative EPSC charge with MAEA (5  $\mu$ M) treatment in wild-type neurons:  $0.57 \pm 0.03$ ,  $n = 4$ ; in CRIP<sub>1a</sub>-transfected neurons:  $0.91 \pm 0.11$ ,  $n = 5$ ;  $P < 0.05$  by unpaired  $t$  test]. To determine whether CRIP<sub>1a</sub> over expression would suppress constitutive inhibition of EPSCs by CB<sub>1</sub>Rs, the effects of rimonabant (100 nM) were examined. However, no effect of this inverse agonist was detected regardless of whether CRIP<sub>1a</sub> was overexpressed (data not shown), as previously reported for non-transfected hippocampal autaptic cultures (Straiker et al., 2012).

To ascertain whether CRIP<sub>1a</sub> transfection interfered more generally with G<sub>i/o</sub>-mediated modulation of neurotransmission, inhibition by the adenosine A<sub>1</sub> receptor agonist cyclopentyladenosine (CPA) was examined, because it was previously found

TABLE 5

$E_{\max}$  and  $EC_{50}$  values of ligand-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in N18TG2 cells with and without stable overexpression or knockdown of CRIP<sub>1a</sub>

Varying concentrations of WIN55,212-2 or MAEA were incubated with membranes prepared from the indicated cell lines in the presence of 20  $\mu$ M GDP and 0.1 nM [<sup>35</sup>S]GTP $\gamma$ S, as described in *Materials and Methods*.  $E_{\max}$  and  $EC_{50}$  values were derived from nonlinear regression analysis of ligand concentration-effect curves. Data are mean values  $\pm$  S.E.M. ( $n = 5-9$ ). Significance of  $E_{\max}$  and  $EC_{50}$  values between cell types were determined by one-way ANOVA with posthoc Newman-Keuls test.

Cell line	WIN55,212-2		MAEA	
	$E_{\max}$	$EC_{50}$	$E_{\max}$	$EC_{50}$
	% Stim	nM	% Stim	nM
WT-N18TG2	45.1 $\pm$ 5.5	53.8 $\pm$ 12.2	40.1 $\pm$ 5.3	497 $\pm$ 197
N18-CRIP <sub>1a</sub> -OX	26.0 $\pm$ 3.5*	82.5 $\pm$ 8.9	30.2 $\pm$ 3.5	594 $\pm$ 178
N18-CRIP <sub>1a</sub> -KD	64.0 $\pm$ 8.5* <sup>§§</sup>	40.9 $\pm$ 8.3	56.2 $\pm$ 5.3* <sup>§</sup>	322 $\pm$ 71

\* $P < 0.05$  different from WT-N18TG2 cells. <sup>§</sup> $P < 0.05$ , <sup>§§</sup> $P < 0.01$  different from N18-CRIP<sub>1a</sub>-OX cells.

Key: N18-CRIP<sub>1a</sub>-OX, N18TG2 cells overexpressing CRIP<sub>1a</sub> (clone 1); N18-CRIP<sub>1a</sub>-KD, N18TG2 cells with siRNA-mediated knockdown of CRIP<sub>1a</sub> (clone 2C).

to robustly inhibit EPSCs in autaptic hippocampal neurons (Straiker et al., 2002). Figure 10C shows that CRIP<sub>1a</sub> overexpression did not interfere with CPA responses [relative EPSC charge after treatment with CPA (100 nM) in control neurons:  $0.27 \pm 0.03$ ,  $n = 10$ ; in CRIP<sub>1a</sub>-transfected neurons:  $0.29 \pm 0.06$ ,  $n = 4$ ]. Together, these results indicate that CRIP<sub>1a</sub> attenuates the inhibition of excitatory synaptic transmission by endocannabinoids, and that this action of CRIP<sub>1a</sub> is selective for modulation of synaptic transmission by CB<sub>1</sub>Rs.

## Discussion

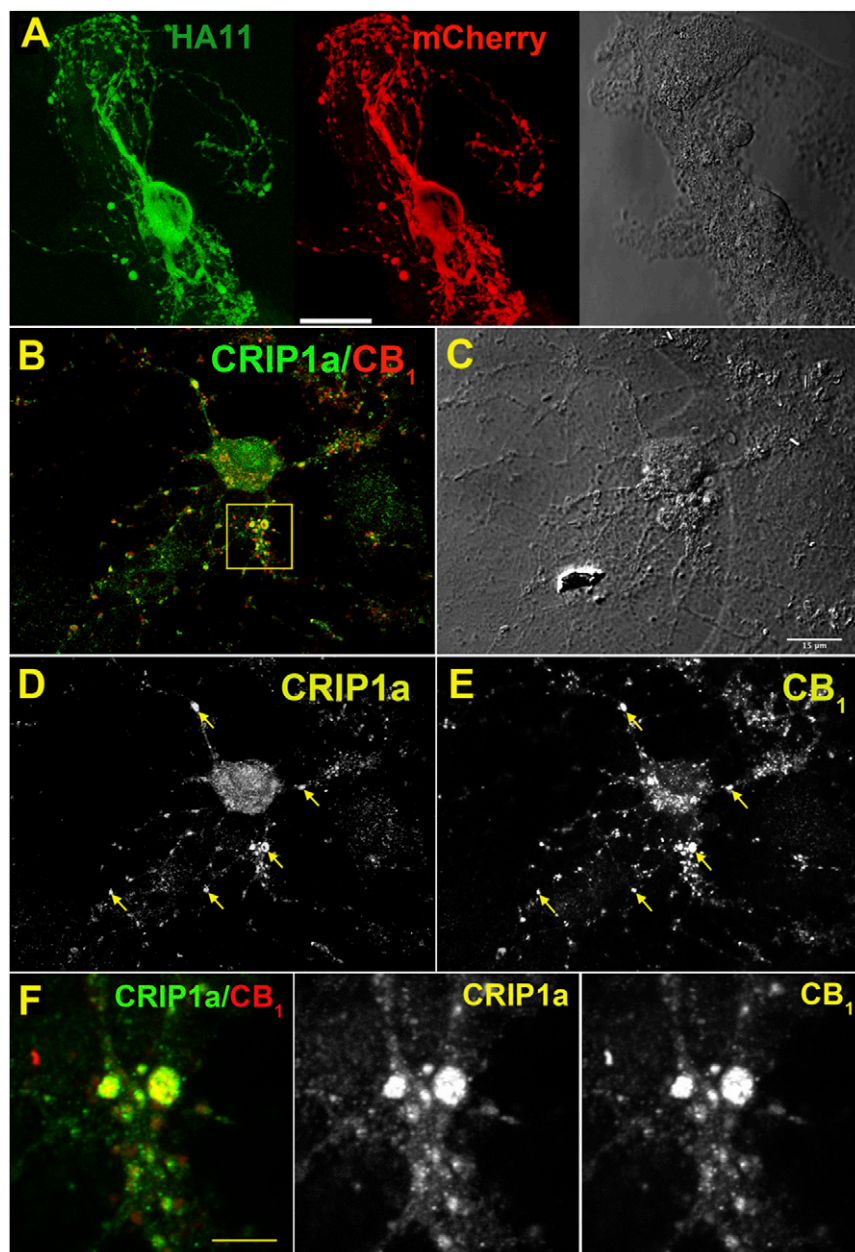
This study extends the findings that CRIP<sub>1a</sub> attenuates constitutive inhibition of Ca<sup>2+</sup> channels by CB<sub>1</sub>Rs in cotransfected SCG neurons (Niehaus et al., 2007). Here we showed that CRIP<sub>1a</sub> attenuated rimonabant-mediated inhibition of basal [<sup>35</sup>S]GTP $\gamma$ S binding in CRIP<sub>1a</sub>-overexpressing CB<sub>1</sub>-HEK cells, suggesting that CRIP<sub>1a</sub> overexpression disrupts constitutive CB<sub>1</sub>R-mediated G-protein activation. CRIP<sub>1a</sub> overexpression also inhibited basal [<sup>35</sup>S]GTP $\gamma$ S binding in CB<sub>1</sub>-HEK cells in the absence of Na<sup>+</sup> but not in the presence of Na<sup>+</sup> or in PTX-pretreated cells, further indicating that CRIP<sub>1a</sub> inhibits CB<sub>1</sub>R constitutive activity. There was an opposing effect of Na<sup>+</sup> on agonist- versus inverse agonist-modulated G-protein activity such that inverse agonism was maximized in the absence of Na<sup>+</sup>, a condition under which PTX-sensitive basal G-protein activity was highest. Conversely, agonist-stimulated G-protein activation was maximized by 100 mM Na<sup>+</sup>, a condition under which PTX-sensitive basal G-protein activation was minimal. CRIP<sub>1a</sub> overexpression suppressed the inverse agonist activity of rimonabant in the absence of Na<sup>+</sup>, whereas CRIP<sub>1a</sub> only suppressed agonist-stimulated G-protein activation with Na<sup>+</sup> present. The absence of endocannabinoids in CB<sub>1</sub>-HEK membranes suggests that CRIP<sub>1a</sub> effects did not result from dampening endocannabinoid tone. Altogether, these results indicate that CRIP<sub>1a</sub> overexpression inhibits constitutive and agonist-stimulated G-protein activity in CB<sub>1</sub>-HEK cells in a manner consistent with the behavior of receptor G-protein complexes under the allosteric modulatory influence of Na<sup>+</sup> (Seifert and Wenzel-Seifert, 2002).

If CRIP<sub>1a</sub> regulates constitutive activity of CB<sub>1</sub>Rs in vivo, then it can be hypothesized to modulate both cellular trafficking of CB<sub>1</sub>Rs and pharmacological responses to inverse agonists. Constitutive activity may be required for CB<sub>1</sub>R internalization and targeting to presynaptic terminals (Leterrier et al., 2004,

2006), although this has been disputed (McDonald et al., 2007). However, confirmation of constitutive CB<sub>1</sub>R activity in the CNS has been elusive. For example, rimonabant concentrations necessary to depress basal G-protein activity in brain are greater than those required to antagonize CB<sub>1</sub> agonist-stimulated activity (Sim-Selley et al., 2001), and high rimonabant concentrations decrease basal G-protein activity in CB<sub>1</sub>R knockout mice (Breivogel et al., 2001). CRIP<sub>1a</sub> overexpression inhibits constitutive CB<sub>1</sub>R activity in CB<sub>1</sub>-HEK cells, so it is possible that CRIP<sub>1a</sub> contributes to the low level of constitutive activity in the brain. This would be consistent with our finding that inverse agonism by rimonabant was not detected in N18TG2 cells, where endogenous CRIP<sub>1a</sub> might suppress constitutive CB<sub>1</sub>R activity. Although siRNA-mediated knockdown of CRIP<sub>1a</sub> did not significantly enhance inverse agonism by rimonabant in N18TG2 cells, this finding could result from the low CB<sub>1</sub>R expression level and the fact that rimonabant was only examined in the presence of 100 mM Na<sup>+</sup> in these cells. Inhibition of basal [<sup>35</sup>S]GTP $\gamma$ S binding by rimonabant in N18TG2 cells is minimal with Na<sup>+</sup> present but detectable after replacement of Na<sup>+</sup> with K<sup>+</sup> (Meschler et al., 2000). Thus, these neural cell models will be useful to investigate CB<sub>1</sub>R inverse agonism and the role of endogenous CRIP<sub>1a</sub> using varying cation concentrations in future studies.

The present study also confirmed that differences in CRIP<sub>1a</sub> expression do not influence total CB<sub>1</sub>R expression levels or ligand binding affinity (Niehaus et al., 2007) in both HEK-293 and N18TG2 cell models, despite the use of different radioligands in each cell line. The finding of similar results with both antagonist/inverse agonist ([<sup>3</sup>H]SR141716A) and agonist ([<sup>3</sup>H]CP55,940) radioligands suggests that CRIP<sub>1a</sub> might influence CB<sub>1</sub>R signaling efficacy without affecting the formation of high-affinity receptor G-protein complexes, which will be addressed in future studies.

The effect of CRIP<sub>1a</sub> on CB<sub>1</sub>R-mediated G-protein activation was dependent on the stoichiometric relationship between CRIP<sub>1a</sub> and CB<sub>1</sub>Rs. In CB<sub>1</sub>-HEK cells, stable CRIP<sub>1a</sub> transfection increased the CRIP<sub>1a</sub>/CB<sub>1</sub>R expression ratio from <0.5 to approximately 7. In N18TG2 cells, which endogenously express both proteins, CRIP<sub>1a</sub> transfection increased the ratio of CRIP<sub>1a</sub>/CB<sub>1</sub>R from approximately 2 to 5. Importantly, CRIP<sub>1a</sub> expression levels in both overexpressed cell lines were lower than in rat cerebellum, suggesting that these cells do not express supraphysiological CRIP<sub>1a</sub> levels relative to native tissues. Whether the ratio of overexpressed CRIP<sub>1a</sub>/CB<sub>1</sub>R is



**Fig. 9.** CRIP<sub>1a</sub> partially colocalizes with CB<sub>1</sub>R in autaptic hippocampal neurons. (A) Micrograph in transfected autaptic hippocampal neuron shows HA11 staining of transfected HA-CRIP<sub>1a</sub> (green) in a mCherry-labeled (red) neuron, indicating that CRIP<sub>1a</sub> is expressed throughout the transfected neuron. Right panel: Nomarski image of the island is shown for reference. Scale bar, 20  $\mu$ m. (B) Endogenous CRIP<sub>1a</sub> (green) and CB<sub>1</sub>R (red) staining in an untransfected autaptic hippocampal neuron (overlap in yellow). (C) Differential interference contrast image corresponding to (B). Scale bar, 15  $\mu$ m. (D) CRIP<sub>1a</sub> and (E) CB<sub>1</sub>R staining from (B). Arrows indicate overlap. (F) Zoom from inset box in (B). Scale bar, 3  $\mu$ m.

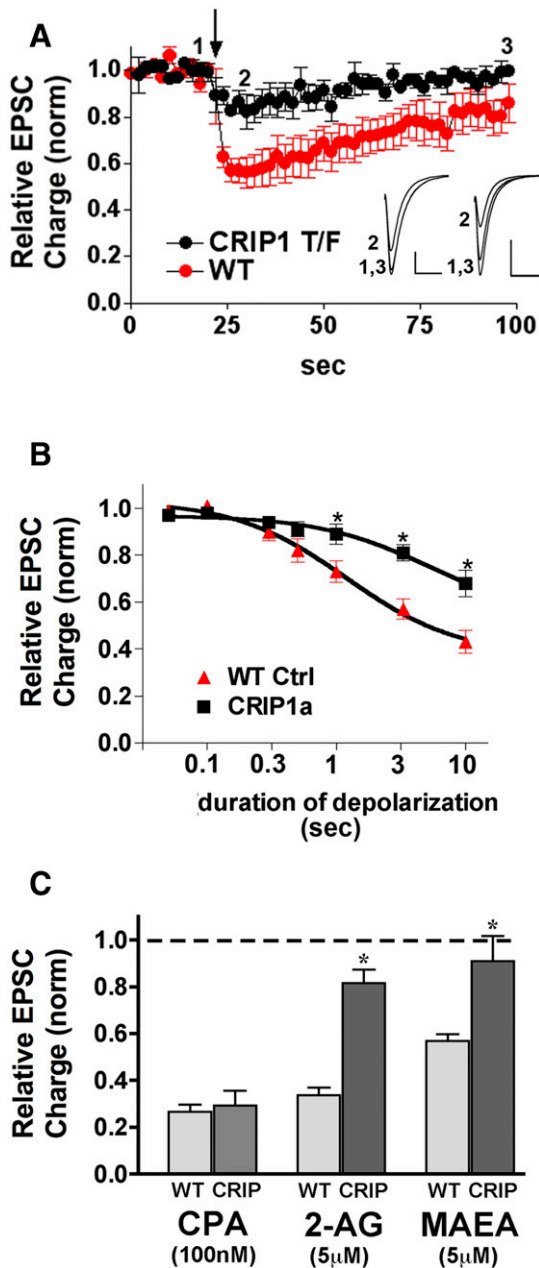
supraphysiological is difficult to ascertain because CRIP<sub>1a</sub> was more widely distributed throughout rat and mouse cerebellum than CB<sub>1</sub>Rs, although colocalization was observed throughout the molecular and granule cell layers.

A major finding of the present study is that CRIP<sub>1a</sub> overexpression attenuated cannabinoid agonist-stimulated G-protein activation in both CB<sub>1</sub>-HEK and N18TG2 cells. This was probably not the result of unnaturally overexpressed levels of CRIP<sub>1a</sub> because the opposite effect—enhancement of agonist-stimulated G-protein activity—was observed with siRNA-mediated knockdown of CRIP<sub>1a</sub> in N18TG2 cells. CRIP<sub>1a</sub> knockdown was not examined in CB<sub>1</sub>-HEK cells because CB<sub>1</sub>Rs were more highly expressed than CRIP<sub>1a</sub> in this model. Although the inhibitory effect of CRIP<sub>1a</sub> overexpression on maximal agonist-stimulated G-protein activation was moderate (~20–40%) in either cell line, a robust effect of CRIP<sub>1a</sub> was observed when comparing N18TG2 cells with CRIP<sub>1a</sub> knocked down versus overexpressed,

whereby up to a 2.5-fold difference in agonist  $E_{max}$  values was observed. These results indicate that CRIP<sub>1a</sub> exerts a dramatic effect on CB<sub>1</sub>R-mediated G-protein signaling under appropriate stoichiometric conditions. Moreover, CRIP<sub>1a</sub> overexpression in autaptic hippocampal neurons attenuated both DSE and inhibition of excitatory synaptic currents by 2-AG and MAEA, demonstrating that CRIP<sub>1a</sub> can regulate one of the most critical functions of CB<sub>1</sub>Rs in neurons. In addition, CRIP<sub>1a</sub> overexpression did not alter synaptic inhibition by an adenosine A<sub>1</sub> agonist, suggesting that CRIP<sub>1a</sub> selectively modulates CB<sub>1</sub>R activity without altering activity of other GPCRs. Altogether, these results suggest that CRIP<sub>1a</sub> could be an important modulator of endocannabinoid signaling in the CNS.

The effects of CRIP<sub>1a</sub> on CB<sub>1</sub>R-mediated G-protein activation were also found to be ligand-dependent in CB<sub>1</sub>-HEK cells. CRIP<sub>1a</sub> overexpression inhibited G-protein activation by agonists of high intrinsic efficacy, including 2-AGE, WIN55,212-2,





**Fig. 10.** Overexpression of CRIP<sub>1a</sub> diminishes CB<sub>1</sub>-mediated DSE in autaptic hippocampal neurons. (A) DSE time-courses for wild-type (WT; red) versus CRIP<sub>1a</sub> transfected neurons (black) in response to 3-second depolarization (arrow). Insets show sample EPSCs from control (1), maximal DSE inhibition (2), and recovery (3) for CRIP<sub>1a</sub>-transfected (left) and WT (right) neurons. (B) "Dose" response for DSE using a range of depolarizations from 50 milliseconds to 10 seconds. The wild-type DSE dose response is shown for comparison. \**P* < 0.05 Bonferroni posthoc test, two-way ANOVA. (C) Bar graph shows relative EPSC charge (1.0 = baseline, no inhibition) after treatment with three drugs under WT and CRIP<sub>1a</sub>-transfected conditions: CPA, 100 nM; 2-AG (5 μM); MetAEA (5 μM). \**P* < 0.05, unpaired *t* test.

HU-210, and CP55,940, but not ligands of lower intrinsic efficacy, including MAEA, levonantradol, and THC. This effect was probably not related solely to chemotype because HU-210 and THC are classic cannabinoids, whereas 2-AGE and MAEA are eicosanoids, yet CRIP<sub>1a</sub> only affected the ligand with highest intrinsic efficacy in each class. It is possible that association of CRIP<sub>1a</sub> with CB<sub>1</sub>Rs is modulated by the presence

of bound ligand in an efficacy-dependent manner. This ligand-selective action of CRIP<sub>1a</sub> was also dependent on cell type, because CRIP<sub>1a</sub> attenuated signaling induced by MAEA in both N18TG2 cells and hippocampal neurons but not CB<sub>1</sub>-HEK cells. Thus, the effects of CRIP<sub>1a</sub> on CB<sub>1</sub>R-G-protein interactions could be dependent on G-protein subtype, which varies among cell types (Atwood et al., 2011). Previous work demonstrated differential association of distinct Gα<sub>i/o</sub> subtypes with CB<sub>1</sub>Rs occupied by different ligands (Mukhopadhyay and Howlett, 2005) and differential abilities of different ligands to activate purified G<sub>i</sub> versus G<sub>o</sub> (Glass and Northup, 1999). In addition, different Gα<sub>i/o</sub> subtypes interact selectively with either the C-terminus or third intracellular loop of the CB<sub>1</sub>R (Mukhopadhyay and Howlett, 2001; Anavi-Goffer et al., 2007), so CRIP<sub>1a</sub> association with the CB<sub>1</sub>R C-terminus (Niehaus et al., 2007) might differentially interfere with G-protein association with these distinct intracellular domains of the CB<sub>1</sub>R. Because the C-terminus serves as a docking site for multiple protein-protein interactions (Howlett et al., 2010; Smith et al., 2010), the effects of CRIP<sub>1a</sub> on CB<sub>1</sub>R-G-protein interactions might depend on both ligand occupancy of the receptor and the presence of additional interacting proteins.

The CB<sub>1</sub>R C-terminus interacts with proteins that mediate desensitization and downregulation. For example, probable G-protein-coupled receptor kinase phosphorylation sites have been identified (Hsieh et al., 1999; Jin et al., 1999; Daigle et al., 2008) and isolated fragments of the CB<sub>1</sub>R C-terminus can bind to β-arrestins (Singh et al., 2011; Bakshi et al., 2007) and GASP1 (Martini et al., 2007). Prolonged agonist exposure downregulates CB<sub>1</sub>Rs expressed in HEK-293 (Shapira et al., 2003) but not N18TG2 cells (McIntosh et al., 1998). Therefore, we examined the effects of prolonged agonist exposure in CB<sub>1</sub>-HEK cells, with and without CRIP<sub>1a</sub> overexpression, on CB<sub>1</sub>R levels and agonist-induced activation of G-proteins. Interestingly, our results showed that CRIP<sub>1a</sub> overexpression interfered with CB<sub>1</sub>R downregulation (degradation) but not desensitization (uncoupling from G-protein activation). These findings suggest that CRIP<sub>1a</sub> might interfere with GASP1 association with CB<sub>1</sub>Rs (Martini et al., 2007, 2010;) or with C-terminal CB<sub>1</sub>R phosphorylation at sites that mediate internalization (Jin et al., 1999; Daigle et al., 2008;) but not at sites that mediate desensitization (Jin et al., 1999; Morgan et al., 2014). Regional differences in CB<sub>1</sub>R downregulation after repeated cannabinoid administration have been observed in the CNS of rodents (Sim-Selley, 2003; Sim-Selley et al., 2006) and humans (Villares, 2007; Hirvonen et al., 2012). Thus, differential colocalization of CB<sub>1</sub>Rs with CRIP<sub>1a</sub> could contribute to regional differences in CB<sub>1</sub>R downregulation, and might thereby influence the development of differential tolerance to distinct pharmacological effects of cannabinoids.

The present study provides evidence that CRIP<sub>1a</sub> attenuates constitutive and agonist-induced G-protein activation by CB<sub>1</sub>Rs in two distinct cell lines, HEK-293 and N18TG2. Additionally, CRIP<sub>1a</sub> inhibits DSE in autaptic hippocampal neurons, suggesting that CRIP<sub>1a</sub> modulates the physiologic actions of endocannabinoids in the CNS. These results indicate that CRIP<sub>1a</sub> is a negative regulator of acute CB<sub>1</sub>R-mediated G-protein signaling. CRIP<sub>1a</sub> also attenuated agonist-induced CB<sub>1</sub>R downregulation, suggesting that it might oppose the development of cannabinoid tolerance. Thus, CRIP<sub>1a</sub> could play an important regulatory role in the endocannabinoid system by differentially modulating acute versus chronic activation of CB<sub>1</sub>Rs.

## Note Added in Proof

A recently published article by some of the coauthors of this paper showed that manipulation of CRIP<sub>1a</sub> expression in N18TG2 cells modulated constitutive and agonist-stimulated extracellular signal-regulated kinase 1/2 phosphorylation by the CB<sub>1</sub>R, and that CB<sub>1</sub>R coupling to G $\alpha_o$  and G $\alpha_3$  was attenuated, whereas coupling to G $\alpha_i1$  and 2 was enhanced, by overexpression of CRIP<sub>1a</sub> (Blume et al., 2015).

## Acknowledgments

The authors thank Hengjun He and Elizabeth Krahn for technical assistance, and Dr. Laura J. Sim-Selley and Erica C. Lyons for critical review of the manuscript.

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*Performed data analysis:* Smith, Blume, Straiker, David, Secor McVoy, Poklis, Cox, Selley.

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