

An Essential Role for Constitutive Endocytosis, but Not Activity, in the Axonal Targeting of the CB₁ Cannabinoid Receptor

Neil A. McDonald, Christopher M. Henstridge, Christopher N. Connolly, and Andrew J. Irving

Neurosciences Institute, Division of Pathology & Neuroscience, Ninewells Hospital & Medical School, University of Dundee, Dundee, Scotland, United Kingdom

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ABSTRACT

In central neurons, the cell-surface distribution of cannabinoid receptor subtype-1 (CB₁) is highly polarized toward axons and is associated with synaptic terminals, in which it is well-positioned to modulate neurotransmitter release. It has been suggested that high levels of constitutive activity mediate CB₁ receptor axonal targeting, leading to domain-specific endocytosis. We have investigated further the mechanisms that underlie CB₁ receptor axonal polarization in hippocampal neurons and found that constitutive activity is not an essential requirement for this process. We demonstrate that the cell-surface distribution of an N-terminally tagged, fluorescent CB₁ receptor fusion-protein is almost exclusively localized to the axon when

expressed in cultured hippocampal neurons. Inhibition of endocytosis by cotransfection with a dominant-negative dynamin-1 (K44A) mutant traps both recombinant and endogenous CB₁ receptors at the somatodendritic cell surface. However, this effect could not be mimicked by inhibiting constitutive activity or receptor activation, either by expressing mutant receptors that lack these properties or by treatment with CB₁ receptor antagonists possessing inverse agonist activity. These data are consistent with a revised model in which domain-specific endocytosis regulates the functional polarization of CB₁ receptors, but this process is distinct from constitutive activity.

The cannabinoid receptor subtype-1 (CB₁) is the most highly expressed G protein-coupled receptor (GPCR) in the mammalian central nervous system and is present at presynaptic terminals and axonal fibers in many brain areas, where it is believed to inhibit neurotransmitter release after agonist binding (Freund et al., 2003). In cultured hippocampal neurons, the cell-surface distribution of endogenous CB₁ receptors is polarized with high levels of expression on GABA-expressing axons, in which they are associated with presynaptic terminals (Irving et al., 2000). A recent study suggested that selective targeting of CB₁ receptors to axons

involves differences in the rate of endocytosis between dendritic and axonal regions, leading to a net accumulation within the axonal compartment, with this process driven by constitutive activation of the CB₁ receptor (Leterrier et al., 2006). Thus, CB₁ receptor antagonists, which are useful therapeutic agents, especially for the treatment of obesity (Van Gaal et al., 2005), may potentially lead to mistargeting of the receptor to the somatodendritic domain and aberrant endocannabinoid function upon cessation of treatment.

In this study, we used N-terminally tagged enhanced green fluorescent protein (GFP)-CB₁ chimeras and immunocytochemical labeling of endogenous surface receptors to further elucidate the mechanisms underlying the polarized distribution of CB₁ receptors in hippocampal neurons. For both endogenous CB₁ receptors and GFP-CB₁ receptor chimeras, polarized surface expression within the axon is

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ABBREVIATIONS: CB₁, cannabinoid receptor subtype-1; GPCR, G protein-coupled receptor; GFP, enhanced green fluorescent protein; MAP2, microtubule-associated protein 2; GAD65, glutamate decarboxylase type 65; AM281, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide; Win55212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate; HU210, (6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol; SR141716A, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; aa, amino acids; HA, hemagglutinin; DMSO, dimethyl sulfoxide; SS, signal sequence; DIV, days in vitro; PBS, phosphate-buffered saline; ANOVA, analysis of variance; D/A, dendritic/axonal; HEK, human embryonic kidney.

achieved by preferential removal from the somatodendritic domain by endocytosis. Using CB₁ receptor antagonists with inverse agonist properties and expression of mutant receptors, we demonstrate that constitutive activity of the receptor is not essential for this effect and that this basal endocytosis represents a distinct cellular process. A revised model is therefore proposed to account for the CB₁ receptor axonal targeting.

Materials and Methods

Constructs and Reagents. Papain and antimicrotubule-associated protein 2 (MAP2) were obtained from Sigma-Aldrich (Poole, Dorset, UK). Antibodies to glutamate decarboxylase type 65 (GAD65) from Chemicon (Hampshire, UK) and the Developmental Studies Hybridoma Bank (GAD-6 clone; Iowa City, Iowa), respectively. Cell culture reagents were sourced from Invitrogen, (Paisley, UK). Indocarbocyanine- and indodicarbocyanine-tagged secondary antibodies were obtained from Stratech Scientific Ltd. (Cambridge, UK), and rabbit anti-GFP was from Abcam (Cambridge, UK). AM281, Win55212-2, and HU210 and forskolin were obtained from Tocris Cookson (Avonmouth, UK). SR141716A was a kind gift from Sanofi-Aventis (Montpellier, France). N-terminal [amino acids (aa) 84–99] CB₁ receptor antibody was obtained from Alomone (Jerusalem, Israel). All restriction enzymes were from Promega (Southampton, UK). Zero Blunt TOPO PCR Cloning Kit and cell culture reagents were obtained from Invitrogen. The original rat CB₁ receptor cDNA and the N-terminal (aa 1–77) CB₁ receptor antibody were kind gifts from Dr. Ken Mackie (University of Washington, Seattle, WA). Cannabinoids were made as 1000× stock solutions in dimethyl sulfoxide (DMSO) and diluted accordingly in culture medium. Dynamin-1 constructs [hemagglutinin (HA)-tagged wild type and K44A] were a kind gift from Dr. Marc Caron (Duke University, Durham, NC) to Dr. Connolly.

Generation of Constructs. GFP was introduced at the N terminus (between residues 25–26) of the cloned, rat CB₁ receptor cDNA and expressed in a pcDNA1/AMP plasmid. Replacement of residues 1 to 25 with an optimized signal sequence (SS, amino acids 1–33; MATGSPSTLLAFGLLCLPWLQEGSARDPPVAT) derived from the human growth hormone (Connolly et al., 1994) enabled efficient surface expression. The human growth hormone-derived signal sequence was amplified by polymerase chain reaction and cloned into pcDNA1 as an HindIII/BamHI fragment to produce SS-pcDNA1. GFP was amplified from pGFP-N1 by polymerase chain reaction and cloned as BamHI/XhoI fragment into SS-pcDNA1 to generate SS-GFP-pcDNA1. Amino acids 26 to 472 of rat CB₁ were amplified by polymerase chain reaction and cloned as a blunt-ended fragment into the TOPO vector. CB₁ 26 to 472 was then digested out of TOPO vector and cloned as a Sall/EcoRI fragment into SS-GFP-pcDNA1 to produce the GFP-CB₁ construct. Point mutation (D164N) and truncation (Δ 14) constructs were generated from this construct and subcloned into a PRK5 vector.

Hippocampal Cell Culture. Primary cultures of rat hippocampal neurons were prepared using a protocol modified from Coutts et al., (2001). In brief, rat pups (1–3 days old) were sacrificed according to United Kingdom Home Office guidelines. Dissected hippocampi were treated with papain (1.5 mg/ml) in HEPES-buffered saline for 25 min at 37°C. Dissociated cells were plated onto coverslips, pretreated with poly(L-lysine) (15 μ g/ml). Cultures were incubated in Neurobasal A medium with B27 supplement (2%) or minimum essential medium with serum replacement 2 (Sigma) in a humidified atmosphere of 5% CO₂ at 37°C. These culture conditions did not affect CB₁ receptor expression and axonal polarity. Cells were used from 3 to 14 days in vitro (DIV), with polarity studies carried out using neurons older than 7 DIV, which exhibit axonal expression of native CB₁ receptors. Treatment with cannabinoid antagonists or DMSO vehicle controls was carried out in culture medium at 37°C.

Neuronal Transfection. Constructs were recombinantly expressed in primary cultures of hippocampal neurons using a CaPO₄ transfection protocol adapted from Jiang et al. (2004). Plasmid DNA (1 μ g of GFP-CB₁ construct per coverslip) was diluted in Tris-EDTA transfection buffer (10 mM Tris-HCl and 2.5 mM EDTA, pH 7.3). CaCl₂ solution (2.5 M in 10 mM HEPES) was then added, drop-wise, to the DNA solution to give a final concentration of 250 mM CaCl₂. This was then added to an equivalent volume of HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 11 mM dextrose, and 42 mM HEPES, pH 7.2). A fraction (1/8th) of the DNA solution was added to the HEPES-buffered transfection solution. This was then vortexed gently for 2 to 3 s, and the precipitate was allowed to develop at room temperature for 30 min, protected from the light. Then, 50 μ l of precipitate was added, drop-wise, to each coverslip, and the cultures were incubated with precipitate for 1 to 3 h in the presence of kynurenic acid (2 mM). Each coverslip was transferred to a fresh well of the 24-well plate containing 1 ml of culture medium with kynurenic acid (2 mM), acidified by equilibration in a 10% CO₂/90% O₂ incubator for 24 h, and the plate was returned to a 37°C/5% CO₂/95% O₂ incubator for 15 to 20 min. Each coverslip was then transferred to a fresh well of the 24-well plate containing reserved, conditioned medium. The cells were then returned to a 37°C 5% CO₂/95% incubator to allow expression of the transfected constructs. Cells were typically analyzed 16 to 48 h after transfection. This procedure resulted in rates of 5 to 15% transfection efficiency.

HEK293 and COS-7 Cell Culture and Transfection. COS-7 cells (American Type Culture Collection, Manassas, VA) and HEK293 cells (Invitrogen) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated neonatal calf serum (Invitrogen), 2 mM glutamine, 1 mM sodium pyruvate, 100 μ g/ml streptomycin, and 100 U/ml penicillin in an atmosphere of 5% CO₂. Suspensions of exponentially growing cells (2 \times 10⁶ cells), detached after trypsin exposure, were transfected by electroporation (400 V, infinity resistance, 125 μ F, Gene Electropulser II; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) in the presence of 10 μ g of DNA. These were then plated onto poly(lysine)-coated coverslips (15 μ g/ml). In some studies, cells growing on coverslips were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were analyzed 12 to 24 h after transfection.

cAMP Immunoassay. Inhibition of forskolin-stimulated cAMP production by the cannabinoid agonist HU210 was assessed using the Catchpoint 96 Fluorescent cAMP immunoassay kit (Molecular Devices, Wokingham, UK). Exponentially growing HEK293 cells were transfected by electroporation with either wild-type CB₁ or GFP-CB₁ and plated onto a 96-well dish coated with poly(D-lysine) (0.15 mg/ml in borate buffer, pH 8.2). Confluent cells were incubated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (100 nM) for 10 min. The stimulation was initiated by the addition of 5 μ M forskolin and the appropriate concentration of HU210 and was incubated for 15 min at 37°C. The reaction was stopped by removing the culture medium and the addition of lysis buffer [diluted to 1× in phosphate-buffered saline (PBS)], after which 40 μ l was removed for the cAMP fluorescent immunoassay. The assay was carried out according to manufacturer's instructions in triplicate.

Cell-Surface Enzyme-Linked Immunosorbent Assay. HEK293 cells were fixed in 3% paraformaldehyde in PBS and then washed twice in 50 mM NH₄Cl (in PBS) and blocked (10% fetal bovine serum, 0.5% bovine serum albumin in PBS) for 1 h. Subsequent washes were performed in block. Cell-surface detection was then carried out using a primary antibody raised against full-length GFP (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) followed by a secondary antibody conjugated to horseradish peroxidase. To quantify the total amount of receptors present, cells from a separate, matched sample of cells from the same transfection were treated with the detergent Triton X-100 (0.5%, 15 min) to permeabilize the cell membranes before treatment with primary antibody. Receptor

expression was determined using a horseradish peroxidase-conjugated secondary antibody and assayed using a spectrophotometer (VersaMax; Molecular Devices), with 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) as the substrate, with detection at 450 nm 30 min after the addition of 0.5 M H₂SO₄. *n* Values represent the number of pooled replicates from three independent experiments, normalized to vehicle control.

Immunofluorescence, Confocal Microscopy, and Trafficking Studies. Neurons were fixed in 3% paraformaldehyde (in PBS), washed twice in 50 mM NH₄Cl (in PBS), and blocked (10% fetal bovine serum and 0.5% bovine serum albumin in PBS) for 30 min. Subsequent washes and antibody dilutions were performed in PBS containing 10% fetal bovine serum and 0.5% bovine serum albumin at room temperature. In some experiments, including those for native CB₁ receptor expression, primary antibody incubations (in HEPES-buffered saline) to label surface receptors were carried out before fixation on live cells. This protocol resulted in a degree of antibody-induced receptor clustering but did not affect the ratio of axonal-dendritic receptor expression. Primary antibodies raised against the N terminus of the CB₁ receptor (dilution 1:50–1:1000; 40–60 min) were used for endogenous CB₁ receptor labeling or against full-length GFP (1:500–1:2000; 60 min) for recombinant GFP-CB₁. Dual-labeling immunohistochemistry was performed after permeabilization (0.5% Triton X-100; 10 min) using monoclonal anti-MAP2 (somatodendritic marker) or anti-GAD65 (GABAergic cell marker) antibodies. For comparison of endogenous cell-surface and intracellular CB₁ receptor-labeling, live cells were labeled with N-terminal (aa 84–99) antibody (1:100), fixed, then probed with secondary antibody (Alexa488 or indocarbocyanine conjugations). The remaining total CB₁ receptor immunoreactivity was assessed after permeabilization (1:500–1000 dilution of anti-CB₁). Live antibody labeling was carried out in the presence of compounds used for cell treatment protocols (DMSO, AM281, and/or Win55212-2). A LSM510 confocal imaging system (Carl Zeiss, Jena, Germany) was used for image acquisition and processing. Images of multiple labeling were obtained in multitracking mode using a 40× water immersion objective. Z-projections were generated from a series of confocal sections (1.5- to 2.5-μm intervals) using a corresponding iris aperture (3–5 μm).

Receptor trafficking studies in neurons and COS7 cells were carried out using a Zeiss LSM510 confocal imaging system at 30°C using a heated stage. Full Z-stack images (2.5-μm sections) were obtained every 10 min using minimal 488 nm excitation (1.9-s scan speed at 521 × 521 pixels; 1–3% maximal argon laser power). For agonist-induced trafficking, low-noise images (7.9-s scan speed) were obtained at the beginning and the end of the experiment (90 min after agonist exposure).

Measurement of Surface Polarity and Data Analysis. The degree of surface polarization for fluorescent CB₁ receptor chimeras and endogenous CB₁ was quantified for reconstructed Z-projections of neurons, analyzed using Zeiss (Laser Sharp) software. The average pixel intensity of surface labeling (indocarbocyanine) was obtained for five regions per axonal or dendritic compartment (20 μm long × 1 pixel wide, GFP channel or intracellular, endogenous CB₁ signal), traced at random. Averaged data with background values subtracted was used to generate a dendritic/axonal (D/A) polarity index. A small population of highly expressing neurons exhibited a cell-surface distribution of GFP-CB₁ that was nonpolarized and were excluded from analysis (<5%). Interneurons expressing endogenous high levels of CB₁ receptor were identified by their increased levels of somatodendritic CB₁ receptor immunoreactivity, which was often vesicular (N-terminal antibody; aa 84–99). For GFP-CB₁-expressing neurons, axons and soma/dendrites were analyzed within the same cell; for endogenous CB₁ receptors, dendritic labeling was compared with axonal fluorescence derived from multiple neurons. Data were compared using appropriate statistical tests (as stated in the results or figure legends using InStat 3; GraphPad Software, San Diego, CA). Significance was noted at the level of *P* < 0.05. Data are presented as

mean ± S.E.M., and *n* values reflect the number of cells or fields (for endogenous CB₁) analyzed from at least two to three different experiments/culture preparations.

Results

Characterization of N-Terminal GFP-CB₁ Receptor Chimeras. The N-terminally tagged GFP-CB₁ functioned normally when expressed in HEK293 cells, with EC₅₀ values for inhibition of forskolin-stimulated cAMP production by the classic agonist HU210 in HEK293 cells similar to those of the wild-type CB₁ receptor, being 2.16 nM (confidence interval 1.28–3.63 nM) for wild-type CB₁ and 0.712 nM (confidence interval = 0.23–2.17) for GFP-CB₁. In addition, this construct exhibited agonist-induced internalization, as measured by cell-surface enzyme-linked immunosorbent assay when expressed in HEK293 cells. Treatment with HU210 (1 μM; *n* = 9) for 2 h significantly reduced cell-surface expression of GFP-CB₁ compared with vehicle-treated cells (41.5 ± 5.6% of vehicle; *n* = 7; unpaired *t* test, two-tailed test; *P* < 0.001).

Distribution of GFP-CB₁ in Cultured Hippocampal Neurons. Next, the functional GFP-CB₁ fusion-protein was transfected into cultured hippocampal neurons (7+ DIV), and its cellular localization was examined in live cells after 16 to 24 h. GFP-CB₁ fluorescence was distributed throughout the cell soma and processes (Fig. 1A) but was excluded from the nucleus with somatodendritic regions exhibiting punctate, vesicular fluorescence. It is noteworthy that axonal

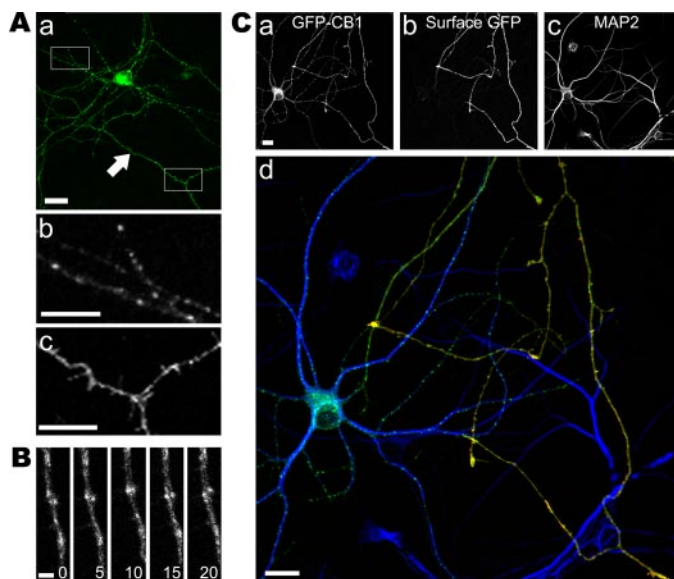


Fig. 1. Surface expression of GFP-CB₁ is restricted to the axon when expressed in cultured hippocampal neurons. Aa, representative Z-projection image of a live, cultured hippocampal neuron (9 DIV) expressing the N-terminally tagged GFP-CB₁ construct, the axon is indicated with an arrow. Zoomed images comparing punctate fluorescence within dendrites (Ab) with more uniform axonal labeling and association with filopodia are illustrated (Ac). Scale bar, 20 μm. B, time lapse recordings of an area of dendrites (confocal section) from a cultured hippocampal neuron (9 DIV) expressing GFP-CB₁. Numbers refer to the time in minutes. Note that puncta are bidirectionally mobile. Scale bar, 2 μm. C, representative Z-projection of a fixed hippocampal neuron (9 DIV) expressing GFP-CB₁ (Ca) and probed for surface expression of GFP (Cb) and intracellular MAP2 (Cc). Note that surface expression of GFP-CB₁ is restricted to the MAP2-negative axon and is more uniformly distributed. Cd, merged color image illustrating GFP-CB₁ fluorescence (green), surface expression (red), and MAP2 labeling (blue). Scale bars, 20 μm.

fluorescence was less punctate, being present throughout axonal shafts and at growth cones. Time-lapse recordings of live, cultured hippocampal neurons expressing GFP-CB₁ demonstrated bidirectional movement of dendritic puncta, showing that they represent mobile, dynamic structures (Fig. 1B). These data suggest that the CB₁ receptor is not actively excluded from the somatodendritic compartment through selective sorting into axon-specific cargo vesicles.

Cell-Surface Polarity of GFP-CB₁ Is Retained in Primary Hippocampal Culture. Cell-surface expression of GFP-CB₁ was assessed using an antibody directed against GFP and was compared with labeling for the somatodendritic marker MAP2 after permeabilization (Fig. 1C). Although GFP-CB₁ fluorescence was clearly present in the cell soma and throughout the full extent of MAP2-positive processes, surface labeling was restricted to the MAP2-negative axon. The polarity index (D/A ratio) was 0.074 ± 0.017 ($n = 12$), reflecting a distribution that is highly polarized toward the axon.

Targeting of GFP-CB₁ to the Axon Is Not Dependent on Hippocampal Neuron Subtype. In the hippocampus, the CB₁ receptor is endogenously expressed on the majority of GABAergic interneurons (Irving et al., 2000). Cell-surface labeling for GFP-CB₁ was detected in transfected mature, cultured hippocampal neurons, and interneurons were subsequently identified by GAD65 expression. The extent of cell-surface polarization of GFP-CB₁ expressed in GABAergic and non-GABAergic neurons was not significantly different ($p > 0.05$) and is therefore not dependent on hippocampal neuron subtype (Fig. 2).

Cotransfection with the Dominant-Negative Dynamin-1 K44A Mutant Leads to a Loss of CB₁ Receptor Cell-Surface Polarity. Previous studies have suggested that the axonal polarization of a C-terminally tagged, fluorescent CB₁ receptor chimera involves dynamin-dependent endocytosis (Leterrier et al., 2006). We have therefore investigated whether this is also the case with our N-terminally tagged GFP-CB₁ construct and endogenous CB₁ receptors using a dominant-negative dynamin-1 mutant (K44A), which inhibits clathrin-dependent endocytosis (Wilbanks et al., 2002).

Mature, cultured hippocampal neurons were cotransfected with GFP-CB₁ and HA-tagged dominant-negative dynamin-1 or wild-type dynamin-1. After 16 to 24 h of expression, the neurons were fixed and the surface distribution of GFP-CB₁ was determined. Cotransfection with HA-tagged dynamin-1 did not alter GFP-CB₁ cell-surface targeting (Fig. 3A). It is striking that, in dominant-negative dynamin-1 (K44A) cotransfected neurons, cell-surface expression of GFP-CB₁ was polarized toward the somatodendritic compartment, although axonal surface expression was still observed (Fig. 3B). The polarity index for GFP-CB₁ in neurons cotransfected with dominant-negative dynamin-1 was significantly different from control neurons ($P < 0.001$), whereas no difference was observed with neurons cotransfected with HA-tagged dynamin-1 ($P > 0.05$; Fig. 3C).

Next, we investigated the effect of inhibiting endocytosis in neurons expressing endogenous CB₁ receptors. Young cultures of hippocampal neurons (3 DIV, when CB₁ receptor cell-surface expression is first observed; Irving et al., 2000) were transfected with the HA-tagged dominant-negative dynamin-1 (K44A) and allowed to express for 48 h before being

fixed at 5 DIV and probed for endogenous CB₁ receptor cell-surface expression. In neurons transfected with the dynamin-1 mutant and endogenously expressing the CB₁ receptor, membrane-associated, cell-surface immunoreactivity for the CB₁ receptor was observed on the cell soma and multiple neurites (Fig. 3D).

Constitutive Activity Does Not Drive CB₁ Receptor Axonal Polarization in Hippocampal Neurons. In some systems, the CB₁ receptor displays constitutive activity (Pertwee, 2005; D'Antona et al., 2006). This is suggested to drive domain-specific endocytosis in cultured hippocampal neurons, in which treatment with an antagonist/inverse agonist alters CB₁ receptor polarity and is associated with an up-regulation of somatodendritic cell-surface expression (Leterrier et al., 2006). We therefore tested for the involvement of constitutive activity in driving GFP-CB₁ receptor axonal targeting using two approaches: either treatment with CB₁ receptor antagonists with inverse agonist properties (AM281 and SR141716A), or expression of recombinant mutant receptors. CB₁ receptors with a D164N point mutation or a 14 amino acid C-terminal truncation ($\Delta 14$) do not undergo agonist-induced internalization (Hsieh et al., 1999; Roche et al., 1999), and the D164N mutant also lacks constitutive activity (Nie and Lewis 2001).

Mature, cultured hippocampal neurons were transfected with GFP-CB₁ and allowed to express for 24 h in the presence of AM281 (1–10 μ M) or SR141716A (1 μ M) or equivalent

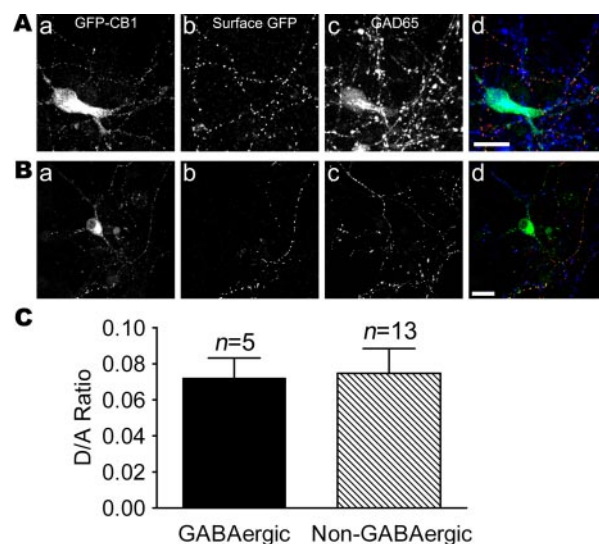


Fig. 2. Surface expression of GFP-CB₁ is restricted to the axon when expressed in both GABAergic and non-GABAergic cultured hippocampal neurons. **A**, surface expression of GFP-CB₁ is restricted to the axon when expressed in GABAergic cultured hippocampal neurons. Representative Z-projection image of a GABAergic hippocampal neuron (8 DIV) expressing GFP-CB₁ (Aa) and probed for surface expression of GFP (Ab) and GAD65 (Ac). Merged image (Ad) illustrating GFP-CB₁ fluorescence (green), surface expression (red), and GAD65 labeling (blue). Scale bar, 20 μ m. **B**, representative Z-projection of a non-GABAergic hippocampal neuron (8 DIV) expressing GFP-CB₁ (Ba) and probed for surface expression of GFP (Bb) and GAD65 (Bc). Merged image (Bd) illustrating GFP-CB₁ fluorescence (green), surface expression (red), and GAD65 labeling (blue). Scale bar, 20 μ m. **C**, the polarity index of dendritic to axonal surface expression (D/A ratio) of GFP-CB₁ was quantified for GABAergic and non-GABAergic neurons expressing the construct. A D/A ratio of <1 reflects a cell-surface distribution that is polarized toward the axon, and a ratio >1 reflects a cell-surface distribution that is polarized toward the dendrites. There was no significant difference (Unpaired t test, two-tailed test; $P > 0.05$).

vehicle-control (1:1000 DMSO). In both vehicle-control and antagonist-treated neurons, cell-surface expression of GFP-CB₁ was still restricted to the axon (Fig. 4A). In addition, shorter periods of exposure (3 h) to a high concentration of antagonists AM281 (10 μ M) or SR141716A (10 μ M) after normal expression of GFP-CB₁ also did not alter polarity (Fig. 4D).

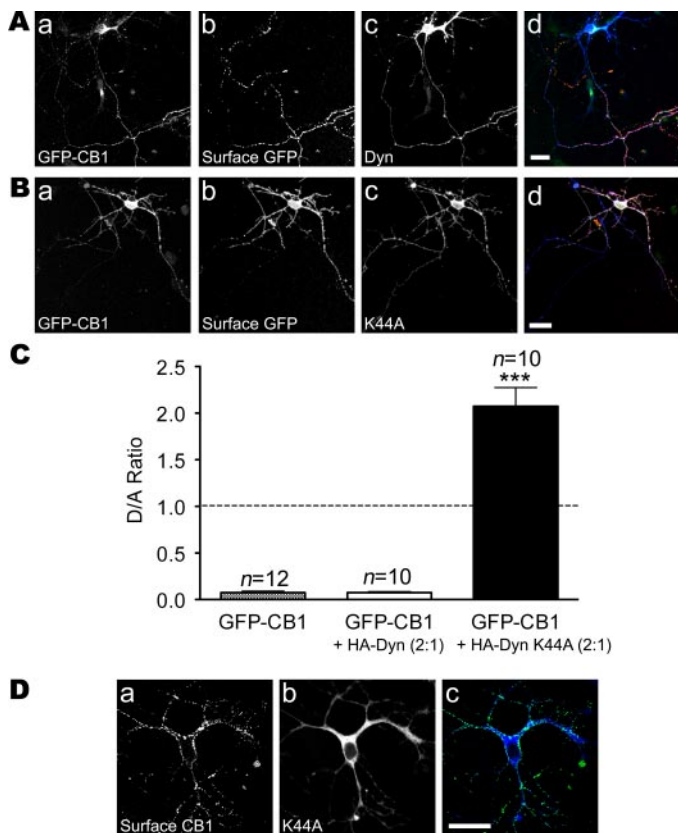


Fig. 3. Preferential endocytosis in the somatodendritic compartment leads to an axonally polarized cell-surface distribution of the CB₁ receptor in cultured hippocampal neurons. A, representative Z-projection of a hippocampal neuron (8 DIV) expressing GFP-CB₁ and HA-tagged, wild-type dynamin-1 (dyn) (Aa) and probed for surface expression of GFP (Ab) and HA-dynamin-1 (Ac). Merged image (Ad) with GFP-CB₁ fluorescence (green), surface expression (red), and HA-dynamin-1 (blue). Note that overexpression of HA-dynamin-1 does not affect axonal polarity. B, cotransfection with an HA-tagged, dominant-negative dynamin-1 (K44A) construct (2:1 ratio) leads to cell-surface expression of GFP-CB₁ on the somatodendritic compartment. Representative Z-projection of hippocampal neuron (8 DIV) expressing GFP-CB₁ (Ba) and probed for surface expression of GFP (Bb) and dominant-negative dynamin-1 (K44A) (Bc). Note that surface GFP-CB₁ fluorescence is present throughout the neuron. Merged image (Bd) with GFP-CB₁ (green), surface expression (red), and HA-dynamin-1 (K44A) (blue). C, the relative intensity of dendritic to axonal surface expression (D/A ratio) of GFP-CB₁ was quantified in neurons expressing GFP-CB₁ alone or GFP-CB₁ cotransfected with either HA-tagged functional or HA-dynamin-1 (K44A). No significant difference was observed between the control and wild-type dynamin-1 (HA-Dyn) cotransfected groups. Cotransfection with the dominant-negative dynamin-1 (HA-Dyn K44A) construct leads to a marked change in the cell-surface polarity of GFP-CB₁ [$P < 0.001$; one-way analysis of variance (ANOVA) with Bonferroni post hoc test]. D, cultured hippocampal neurons (3 DIV) were transfected with dominant-negative dynamin-1 and allowed to express for 48 h before detecting surface expression of endogenous CB₁ receptor at 5 DIV with a N-terminal antibody. A representative Z-projection of a CB₁ positive neuron is illustrated. Da, membrane-associated CB₁ surface labeling on the soma and dendrites. Db, labeling for the HA-dynamin-1 (K44A). Dc, merged image with CB₁ receptor surface labeling (green) and HA-dynamin-1 (K44A) (blue). Scale bars, 20 μ m.

Next, we studied the effects of mutant constructs lacking agonist-induced internalization/constitutive activity. In control experiments with GFP-CB₁ expressed in COS7 cells, Win55212-2 treatment resulted in the appearance of pronounced perinuclear, vesicular fluorescence within the cytoplasm after 90-min exposure (30°C), which is consistent with agonist-induced trafficking (Hsieh et al., 1999). However, no trafficking was observed in cells expressing CB₁(D164N) or the GFP-CB₁ (Δ 14) mutants after 90-min exposure to 100 nM Win55212-2 (Fig. 4B). When expressed in neurons, the mutant constructs GFP-CB₁(D164N) ($n = 8$) and GFP-CB₁(Δ 14) ($n = 11$) also exhibited normal axonal targeting with respect to GFP-CB₁ (Fig. 4C). Thus, the polarity indexes for GFP-CB₁ in vehicle and AM281-treated cells and for the GFP-CB₁ mutants were not significantly different ($P > 0.05$; Fig. 4E).

Treatment with CB₁ receptor antagonists also had no effect on the axonal polarization of endogenous CB₁ receptors detected using an N-terminal antibody. Exposure of cultures to AM281 (10 μ M; Fig. 5) or SR141716A (1 μ M; Fig. 5) for 17 to 18 h did not result in a significant up-regulation of endogenous somatodendritic CB₁ receptor cell-surface expression, and CB₁ receptor surface immunoreactivity remained highly polarized toward the axon. Prolonged treatment with agonist (Win55212-2; 17 h) resulted in the appearance of pronounced punctate labeling within somatodendritic regions (Coutts et al., 2001) and a marked reduction in cell-surface labeling within the axon and dendrites (Fig. 5D). This was associated with a small increase in the polarity index, reducing axonal surface labeling to a slightly greater extent than soma/dendrites (Fig. 5F). The observation that weak dendritic cell-surface labeling was also reduced in the presence of Win55212-2 suggests that low levels of surface receptor expression can be detected within this compartment. All of the effects of Win55212-2 were prevented in the presence of AM281 (10 μ M) (Fig. 5).

Discussion

In cultured hippocampal neurons, cell-surface immunoreactivity for the CB₁ receptor is markedly polarized toward the axon (Irving et al., 2000; Leterrier et al., 2006). In this study, we used an N-terminally tagged GFP-CB₁ receptor fusion-protein together with immunohistochemically labeled endogenous CB₁ receptors to investigate the mechanisms underlying CB₁ receptor axonal targeting. We find that CB₁ receptors are delivered to both axonal and somatodendritic plasma membranes but that surface polarity is achieved by selective, constitutive endocytosis/basal receptor removal. However, this process does not require constitutive receptor activity.

Axonal Targeting. As with endogenous CB₁ receptors, the cell-surface expression of recombinant GFP-CB₁ is highly polarized toward the axon when expressed in mature, cultured hippocampal neurons. Although hippocampal cultures contain a mixed population of cells, endogenous CB₁ receptors are expressed at high levels within a subset of GABAergic interneurons (Irving et al., 2000). However, the cell-surface expression of GFP-CB₁ was independent of the subtype of hippocampal neuron expressing the receptor. This is consistent with the receptor containing axonal-surface targeting information and the conservation of trafficking pathways between the different hippocampal cell types.

Preferential Endocytosis of the CB₁ Receptor in the Somatodendritic Compartment Drives Its Functional Polarization. Intracellular GFP-CB₁ receptors were observed throughout the neuron, suggesting that active exclusion of the CB₁ receptor from the dendritic compartment does not occur. Consistent with recent work using hippocampal cultures (Leterrier et al., 2006), preferential endocytosis of the CB₁ receptor from the somatodendritic plasma-membrane underlies axonally polarized cell-surface CB₁ expression. This was demonstrated using transfection with a dominant-negative dynamin-1 mutant, which competitively inhibits clathrin-mediated endocytosis. It is noteworthy that endogenous dynamin levels did not seem to be limiting in the somatodendritic endocytosis of the CB₁ receptor, because overexpression of wild-type dynamin-1 did not decrease the polarity index of GFP-CB₁. In addition to demonstrating a change in recombinant surface receptor expression by inhibiting endocytosis, we also demonstrated this effect for native CB₁ receptors expressed in interneurons. A similar role of endocytosis in the axonal cell-surface polarization of vesicle-associated membrane protein 2 (Sampo et al., 2003) and neuroglia cell adhesion molecule (Sampo et al., 2003; Wisco et al., 2003) has been proposed. It is likely that proteins polarized to the axon will, at least initially, also be trafficked to the soma and dendrites (Stowell and Craig, 1999), and domain-specific endocytosis seems to be increasingly recog-

nized as important in limiting inappropriate surface expression of axonal proteins within this region (Wisco et al., 2003).

The precise mechanisms that underlie the preferential endocytosis of the CB₁ receptor within the somatodendritic compartment are unclear at present. Differences in the internalization machinery may play a role; for example, the expression of dynamin subtypes varies between axonal and somatodendritic compartments (Gray et al., 2003). Endocytosis within axons may also be spatially restricted to synapses, with nonsynaptic CB₁ receptors more resistant to this (Leterrier et al., 2006). Alternatively, specific anchoring proteins present in axons may bind CB₁ receptors and stabilize them within the plasma membrane. Studies in polarized cell model systems suggest that anchoring to scaffolding proteins and/or the cytoskeleton underlies the retention of membrane proteins at specialized plasma membrane domains. For example, the integrity of the actin cytoskeleton is required for the retention of resident apical proteins at the apical plasma membrane in polarized WIF-B hepatic cells, and it is proposed that actin-based scaffolds actively exclude these proteins from endocytosis (Tuma et al., 2002).

Constitutive Activity Is Not a Prerequisite for Axonal Targeting. Previous studies in HEK293 cells suggest that the CB₁ receptor exhibits constitutive activity, which leads to a pronounced intracellular localization under control conditions by driving receptor endocytosis (Leterrier et al.,

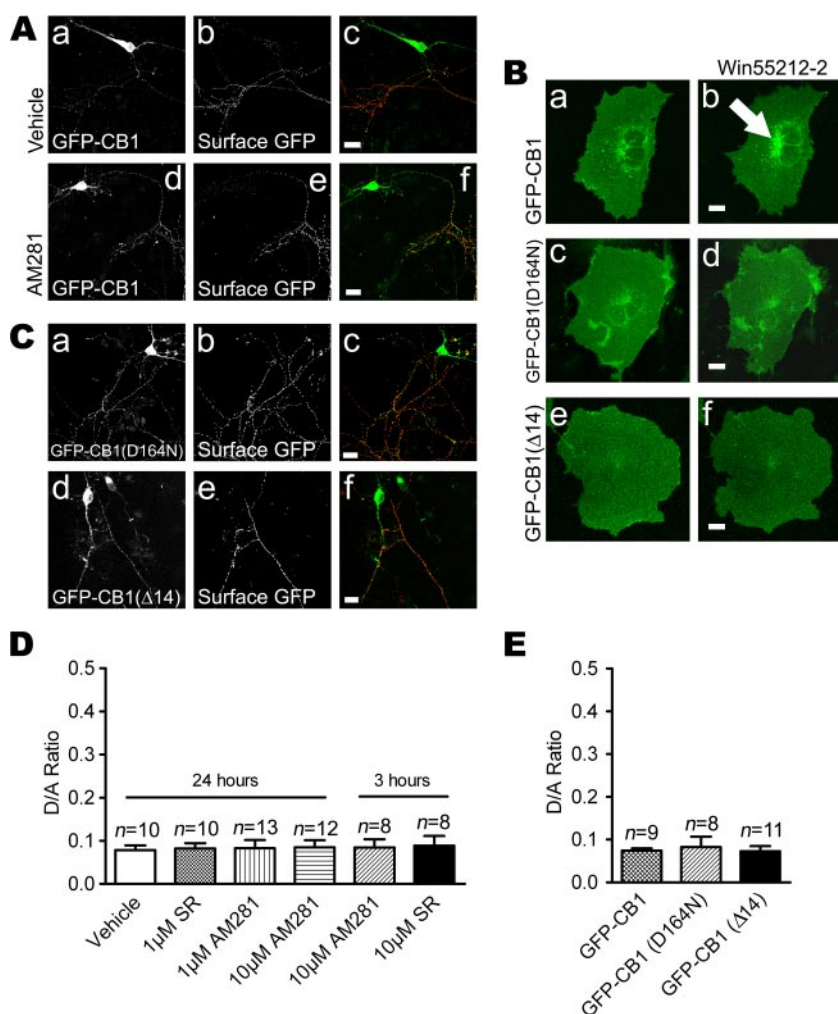


Fig. 4. Constitutive activity is not required for removal of the CB₁ receptor from the somatodendritic cell-surface. A, antagonist treatment does not affect the cell-surface polarity of GFP-CB₁ when it is expressed in cultured hippocampal neurons. Representative Z-projection images of neurons (8 DIV) transfected with GFP-CB₁ and allowed to express in the presence of either vehicle (1:1000 DMSO; A, a, b, and c) or 10 μM AM281 (A, d, e, and f). GFP-CB₁ fluorescence (A, a and d) is present throughout the neuron, but surface expression of GFP-CB₁ (A, b and e) is restricted to the axon in both treatments. Merged images (A, c and f) with GFP-CB₁ (green) and surface labeling (red). B, agonist-induced trafficking of mutant constructs was assessed in COS7 cells. Cells expressing GFP-CB₁ (B, a and b) GFP-CB₁(D164N) (B, c and d) GFP-CB₁(Δ14) (B, e and f) were exposed to Win55212-2 (100 nM) for 90 min. Note that agonist-induced trafficking was only observed in cells transfected with GFP-CB₁ (arrow). B and C, the cell-surface polarity of GFP-CB₁ mutants was assessed in hippocampal neurons. A representative Z-projection image of a neuron (8 DIV) transfected with GFP-CB₁ (D164N) (C, a–c) GFP-CB₁ (Δ14) (C, d–f) and probed for surface expression (C, b and e). Merged image (C, c and f) illustrating GFP-CB₁ (green) and surface labeling (red). Note that in these studies, neurons were probed for surface expression using an antibody directed against GFP before fixation, which resulted in a small degree of receptor clustering. However, this labeling was still restricted to the axon. Scale bars, 20 μm. D and E, the D/A ratio for surface GFP-CB₁ was quantified for different antagonist treatment protocols (D) and GFP-CB₁ mutants (E). No significant difference in the D/A ratio for any of the antagonist treatments or mutant receptors was observed ($P > 0.05$; one-way ANOVA with Bonferroni post hoc test). Scale bars, 20 μm for neurons (A and C) and 50 μm for COS7 cells (B).

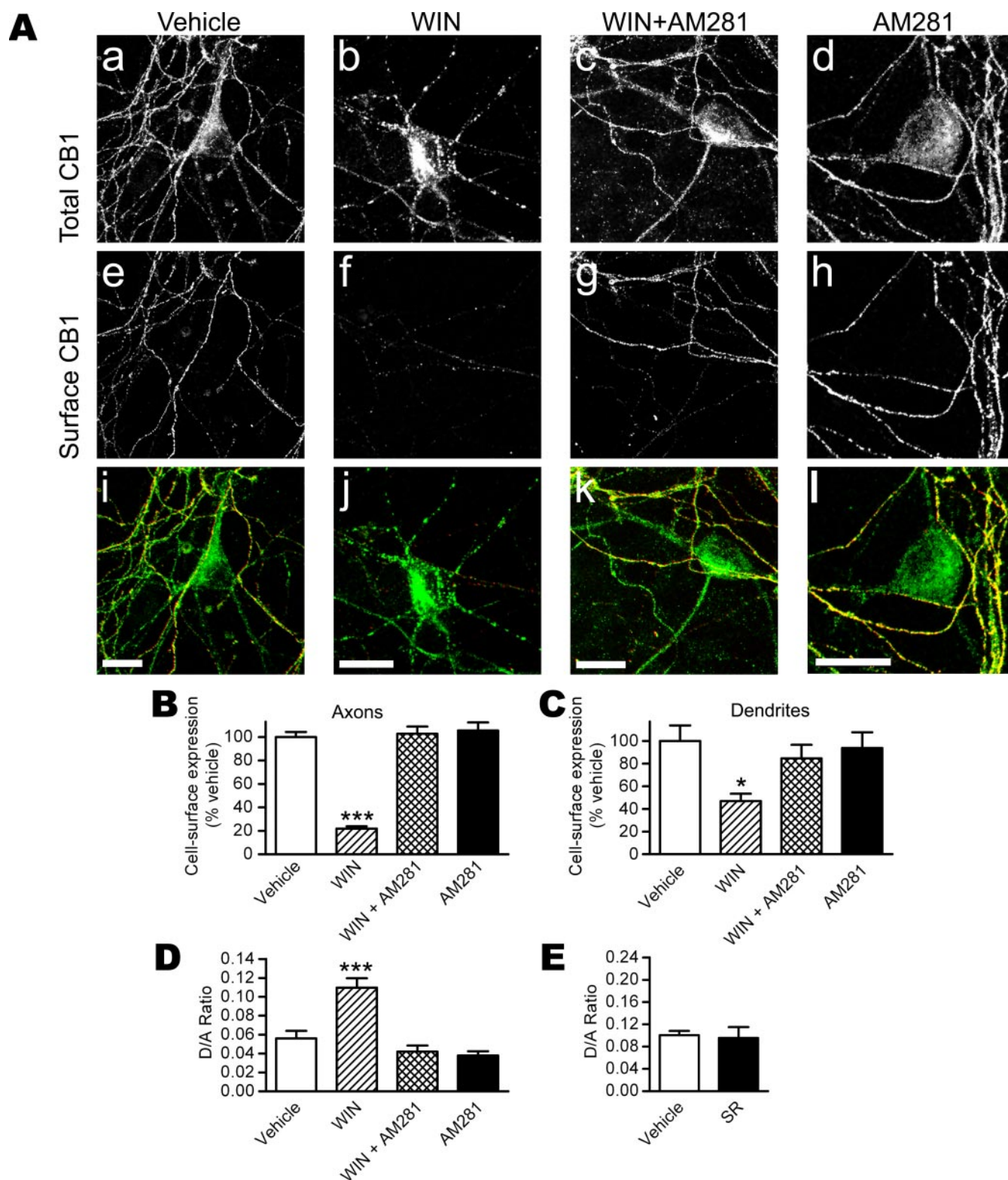


Fig. 5. Antagonist treatment did not alter surface expression of endogenous CB₁ receptors. Representative confocal images (Z-projections) of CB₁ receptor-positive neurons from a culture treated for 17 h with vehicle, (A, a, e, and i; $n = 16$), Win55212-2 (100 nM; A, b, f, and j; $n = 12$), Win55212-2 (100 nM) and AM281 (10 μM) (A, c, g, and k; $n = 16$) or AM281 (10 μM) (A, d, h, and l; $n = 9$) alone. Cultures were then probed for CB₁ receptor surface expression (A, e, f, g, and h) and then for additional CB₁ receptor labeling after fixation and permeabilization (Total CB₁; A, a, b, c, and d). Merged images illustrate CB₁ receptor surface expression (green) and total CB₁ (green) (A, i, j, k, and l). Scale bars, 20 μm. Cell-surface CB₁ expression levels were quantified for axons (B) and dendrites (C) and D/A ratios for surface endogenous CB₁ (D) were determined for the different treatment protocols. No significant difference in the D/A ratio was observed with AM281 ($P > 0.05$); however, treatment with Win55212-2 decreased surface expression on dendritic regions and on the axon, resulting in a slight change in polarity toward soma and dendrites ($P < 0.05$). E, D/A ratios for surface endogenous CB₁ were quantified after 18-h exposure to SR141716A (SR; 1 μM) relative to vehicle control. No significant difference in the D/A ratio was observed ($P > 0.05$; one-way ANOVA with Bonferroni post hoc test).

2004; D'Antona et al., 2006). Leterrier et al. (2006) have suggested that this is analogous to endocytosis of the CB₁ receptor within the somatodendritic compartment of hippocampal neurons. However, in contrast to this, we find that constitutive activation of the CB₁ receptor is not required for its axonal polarization. Constitutive activity is generally believed to reflect the periodic spontaneous entry of the receptor into its active conformation in the absence of agonist (Bond and IJzerman, 2006). In certain systems, tonic activation can drive constitutive endocytosis of the CB₁ receptor via a mechanistically similar process to agonist-induced internalization (Leterrier et al., 2004; D'Antona et al., 2006). However, the CB₁ receptor does not exhibit constitutive activity in all systems, and an increasing number of studies suggest that it may not be tonically active in central neurons (Coutts et al., 2001; Savinainen et al., 2003; Hentges et al., 2005; Zhu and Lovinger, 2005; Neu et al., 2006), and this may reflect the expression of modulatory proteins that block this activity.

It was proposed by Leterrier et al. (2006) that the CB₁ receptor antagonist/inverse agonist AM281 can change the polarity of CB₁ expression by trapping constitutively active receptors at the somatodendritic plasma membrane. However, we saw no evidence for this effect with either AM281 or SR141716A, even at high concentrations (10 μ M) at which inverse agonism will be more apparent (Pertwee, 2005). Indeed, we have reported previously that overnight treatment with SR141716A does not up-regulate native CB₁ receptor expression, and the cellular distribution of surface receptors remained unchanged (Coutts et al., 2001). This observation was corroborated in the present study for both endogenous and recombinant CB₁ receptors. Because incubation with CB₁ receptor antagonists does not disrupt the CB₁ receptor cell-surface polarity, this also suggests that it is not dependent on the basal release of endocannabinoids. Further evidence for a mechanism distinct from constitutive activity comes from our findings with mutant chimeras that are reported to prevent agonist-induced endocytosis (Hsieh et al., 1999; Roche et al., 1999) and constitutive activity (D164N; Roche et al., 1999; Nie and Lewis, 2001) but do not affect CB₁ receptor surface polarity.

Our data are consistent with a model in which the constitutive endocytic removal of the CB₁ receptor from the somatodendritic plasma membrane occurs through a cellular process distinct from constitutive activation-driven endocytosis. As such, this is likely to involve motifs/conformational states different from those used by agonist-induced internalization and may also involve different endocytic proteins. The significant down-regulation of dendritic CB₁ cell-surface expression by agonist in the absence of a reciprocal up-regulation by antagonist further suggests that the receptor is constitutively removed from the plasma membrane independently of receptor activation. It is interesting that differences in structural or conformational requirements for constitutive and agonist-promoted endocytosis have been identified in other GPCR systems (Whistler et al., 2002; Waldhoer et al., 2003).

The marked contrasts between the present study and that of Leterrier et al. (2006) are probably multifactorial in their explanation. For example, truncations of the C-terminal tail of the CB₁ receptor increases constitutive activity (Nie and Lewis, 2001), and tagging the CB₁ receptor at its C terminus

(Leterrier et al., 2004) might prevent this effect, leading to higher levels of constitutive activity. Differences in the developmental age of the cultures or culture conditions (including endocannabinoid contaminants or by inducing their generation) could also influence the apparent expression of constitutively precoupled receptors.

Conclusions

Our data are consistent with a revised model in which the CB₁ receptor is delivered to the cell-surface membrane in both the axonal and somatodendritic compartments but is removed from the somatodendritic plasma membrane by constitutive endocytosis, which is a process that is distinct from agonist-driven endocytosis. Moreover, constitutive activation of the receptor does not drive endocytosis in cultured hippocampal neurons or influence axonal polarity. Antagonists of the CB₁ receptor are of considerable therapeutic interest, and treatment is likely to be long-term (Van Gaal et al., 2005). Our findings suggest that such compounds are unlikely to lead to a marked redistribution of CB₁ receptors from their correct functional sites within the brain.

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Address correspondence to: Dr. Andrew Irving, Neurosciences Institute, Division of Pathology and Neuroscience, Ninewells Hospital and Medical School, University of Dundee, Dundee, DD1 9SY. E-mail: a.j.irving@dundee.ac.uk
