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Cannabinoid Receptor 1 trafficking and the role of the intracellular pool: Implications for therapeutics

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ABSTRACT

Cannabinoid Receptor 1 (CB_1), an abundant G-protein coupled receptor in the CNS, is currently of significant interest as a therapeutic target. Although the cellular control of receptor trafficking is intimately linked with drug effects, CB_1 trafficking is poorly defined in the current literature and conflicting evidence exists as to whether CB_1 should be classified as a recycling, degrading or "dual-fate" receptor. Of particular interest is the widely noted intracellular pool which has been speculated to form part of a constitutive internalization and recycling pathway. This study performs a detailed quantification of CB_1 trafficking in four cell lines, one of which expresses CB_1 endogenously. We demonstrate that, contrary to previous reports, CB_1 does not recycle following constitutive or agonist-induced internalization but instead exhibits a primarily degradative phenotype. Further, our data suggest that the intracellular pool does not contribute to cell surface re-population. These findings have significant implications for the interpretation of CB_1 biochemical studies and the design and application of cannabinoid therapeutics.

1. Introduction

Cannabinoid Receptor 1 (CB_1) is one of the most abundant G-protein coupled receptors (GPCRs) in the mammalian CNS and is also expressed at various sites in the periphery [1]. As the mediator of endocannabinoid (eCB) effects on a variety of brain and systemic functions, with postulated roles in a number of neurological and immune disorders (reviewed in [2]), CB_1 is the target of a number of therapeutics recently approved or currently in clinical trials (e.g. Marinol, Cesamet, Sativex).

For the majority of receptor-mediated drug treatments, the quantity of receptors present at the cell surface is a fundamental determinant of the potential cellular response. Considerable research in recent times has therefore focused on understanding GPCR intracellular trafficking. Following internalization, receptors can enter divergent post-endocytic pathways that confer distinct effects on cell signaling. Trafficking of receptors to degradative pathways leads to a prolonged loss of responsiveness (e.g. [3]) whereas the recycling of receptors back to the surface allows the cell to remain responsive to further activation (reviewed in [4]). Recent studies have revealed that the delivery of receptors to particular pathways is highly organized (e.g. [5–7]) and associated interacting proteins may present drug targets through which receptor localization and activity, and consequently cellular responses, could be controlled [8].

In the brain, CB_1 is expressed at the pre-synaptic cell membrane of axon terminals (e.g. [9,10]). Plasma membrane expression in primary neuronal cultures is associated with axonal processes [11–13], and is also present in endogenously-expressing [14,15] and transfected [16–20] immortalized cell lines. However, in the brain (e.g. [9,21,22]), as well as in each of these cell systems, a significant proportion of CB_1 is located in an "intracellular pool" in the cytoplasm. In both transfected cells and those that endogenously express CB_1, this intracellular pool displays only minimal co-localization with protein synthesis-associated organelles [18,23]. This observation, combined with the ability of the receptor to constitutively endocytose [18] and correlation of results with other receptors that exhibit similar phenotypes (e.g. [24–26]) have led to

Abbreviations: AM, AM 251; CB_1, Cannabinoid Receptor 1; CHO, Chinese hamster ovary-K1; CHX, cycloheximide; Con A, Concanavalin A; D_1, Dopamine Receptor 1; DMEM, Dulbecco's Modified Eagle Medium; eCB, endocannabinoid; ER, endoplasmic reticulum; FBS, fetal bovine serum; GPCR, G-protein coupled receptor; HA, hemagglutinin; HEK, human embryonic kidney-293; HU, HU 210; NFM, non-fat milk; PBS-T, PBS with 0.2% Triton X-100; PNGase, peptide-N-glycosidase F; RT, room temperature; SFM, serum-free media; SR, SR 141716A; TBS, tris-buffered saline; TBS-T, TBS with 0.05% Tween; WIN, WIN 55212-2.

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the inference that this intracellular pool serves as a reservoir of endocytic origin. This reservoir may function as a source from which surface CB_1 is replenished to replace internalized receptor [18], suggesting that CB_1 exhibits a recycling phenotype.

In contrast to this recycling hypothesis, downregulation of CB_1 following chronic agonist stimulation has also been widely reported (reviewed in [27]), and recently GASP-1 [19,28] and AP3 [23], adaptor proteins associated with sorting and delivery of receptors to lysosomes, were demonstrated to interact with CB_1. Furthermore, interruption of the GASP-1 interaction with a dominant-negative mutant prevented degradation [19]. Thus, there is evidence for both recycling and degradation of CB_1.

In this study, we have utilized a high-throughput receptor quantification technique [29] to perform a detailed characterization of CB_1 intracellular trafficking and to investigate the role of the intracellular pool in four cell lines, one of which expresses CB_1 endogenously. We demonstrate that CB_1 exhibits a primarily degradative phenotype and that the widely noted intracellular pool does not contribute to cellular re-sensitization.

2. Materials and methods

2.1. Cell culture

Cell culture and molecular biology reagents were from Invitrogen (Carlsbad, CA) unless otherwise stated. Human embryonic kidney-293 (HEK) cells (ATCC #CRL-1573) were transfected with rat CB_1 chimerized with a single hemagglutinin (HA) tag [30], rat CB_1 chimerized with a single FLAG tag, or human Dopamine Receptor 1 (D_1) with three HA tags. AtT-20 and Chinese hamster ovary-K1 (CHO; ATCC #CRL-9618) cells were transfected with human CB_1 chimerized with three HA tags. CB_1 and D_1 constructs (Missouri S&T cDNA Resource Center, www.cdna.org, #CNR01LTN00 and #DRD010TN00) were sub-cloned via KpnI/PmeI or KpnI/XbaI restriction sites (respectively) from pcDNA3.1(+) to pEF4 A. ScaI-linearized DNA was transfected into cells with Lipofectamine™ 2000. Clonal populations stably expressing each receptor were selected. All HA epitope tags were chimerized at the extreme amino termini of the receptor sequences.

Cells were maintained at 37 °C/5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) except the CHO cells which were maintained in DMEM-F12 with 10% FBS. Transfected lines were cultured with 250 μg/mL Zeocin and the Neuro-2a (ATCC #CCL-131) cell media was buffered with 25 mM HEPES pH 7.4.

Cells were seeded at an appropriate density to reach 70–80% confluence by the end of the experiment on Poly-L-Lysine (0.2 mg/mL in PBS, Sigma-Aldrich, St Louis, MO) treated 96-well plates or 35 mm culture dishes (for Western blotting) and allowed to recover overnight. Plates or slides were instead treated with Poly-D-Lysine (0.05 mg/mL in PBS, Sigma-Aldrich) if cells were to be treated with trypsin (details below), or for confocal imaging experiments when cells were plated on multi-chamber glass culture slides (BD Biosciences, San Jose, CA).

2.2. Receptor trafficking assays

Unless stated otherwise, incubations were performed at 37 °C and compounds or antibodies were diluted in serum-free media (SFM; DMEM or DMEM-F12 with 5 mg/mL BSA (ICPbio, Auckland, NZ)). All vessels used to dilute or dispense compounds or vehicle were silanized prior to use (Coatasil, Ajax Finechem, Sydney, NSW).

At the start of each assay cells were equilibrated in SFM for 15 min. When surface receptors were to be labeled with antibody prior to treatment, cells were incubated for 30 min (unless stated otherwise) with mouse monoclonal anti-HA11 antibody (#MMS-101P, Covance, Berkeley, CA) diluted 1:500 or rabbit polyclonal anti-CB_1 N-terminal antibody [22,31] diluted 1:250 and washed twice with SFM prior to the addition of compounds. The anti-HA11 antibody was used for all experiments with HEK, AtT-20 and CHO cells, while the anti-CB_1 antibody was used for all experiments with Neuro-2a cells. An anti-FLAG primary antibody (M1 mouse monoclonal, #F3040, Sigma-Aldrich) was utilized for one experiment with FLAG-rCB_1 HEK cells.

The details of cell treatments are noted in the text. Unless otherwise stated, compounds were from Sigma-Aldrich and were suspended in absolute ethanol. Compounds applied were: AM 251 (AM; Tocris Bioscience, Ellisville, MO), HU 210 (HU; Tocris Bioscience), WIN 55212-2 (WIN; Tocris Bioscience), SR 141716A (SR; National Institute on Drug Abuse, Rockville, MD), dopamine (prepared fresh for each experiment in H_2O), SCH 23390, Concanavalin A (Con A; suspended in 1 M NaCl), monensin and cycloheximide (CHX). All conditions were matched with appropriate vehicle and washing controls for the compounds applied (usually absolute ethanol diluted 1:5000, maximum 1:2500). A minimum of three replicate wells per condition were included in each experiment.

At the conclusion of stimulations, plates were placed on ice to prevent any further receptor trafficking. Cells were incubated with primary antibody (at the concentrations above) to assay net surface receptor, or secondary antibody (1:300; details below) to assay surface primary antibody-bound receptor (in live labeling protocols) for 30 min at room temperature (RT). When intracellular receptors were to be assayed, cells were incubated with 0.05% trypsin-EDTA·4Na (pH 7.4, Invitrogen) for 1 min at RT; control cells for comparison were incubated with 0.2 g/L EDTA·4Na alone (Versene, pH 7.4, Invitrogen). Cells were washed twice with SFM, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (10 min at RT) and washed three times with PBS.

2.3. Immunocytochemistry

For experiments assaying total and/or intracellular receptor, fixed cells were incubated with anti-HA11 antibody diluted 1:1000 or anti-CB_1 antibody diluted 1:500 in immunobuffer (PBS with 1% normal goat serum, Invitrogen, and 0.4 mg/mL Thiomersal, Merck, Darmstadt, Germany) with 0.2% Triton X-100 overnight at 4 °C and subsequently washed once for 10 min in PBS with 0.2% Triton X-100 (PBS-T). Prior to incubation with Gα subunit antibodies diluted 1:500 (Calbiochem #371720 and #371726), fixed cells were treated with 90% methanol for 10 min at −20 °C.

Experiments were subsequently incubated with Alexa Fluor® 488 or 594-conjugated anti-mouse or anti-rabbit IgG raised in goat (Invitrogen) diluted 1:400 in immunobuffer for 3 h at RT, or overnight at 4 °C. Cells were then washed once for 10 min with PBS-T, stained with Hoechst 33258 (8 μg/mL in PBS-T, 10 min at RT, Invitrogen) and washed twice again with PBS-T.

Glass slides were mounted in AF-1 (Citifluor, Leicester, UK) with a #1.5 coverslip. Confocal images were obtained on a Leica TCS SP2 system with 63× objective lens (Leica HCX PL APO, NA 1.32), Airy 1 pinhole, and line averaging (8). Widefield images were acquired on a Discovery-1™ inverted microscope (see below) with a 40× objective lens (Nikon Plan Fluor, NA 0.6). Images are representative of at least three independent observations.

2.4. Assay quantification

The image acquisition and analysis approach utilized has been discussed in detail previously [29]. Briefly, a Discovery-1™ (Molecular Devices, Sunnyvale, CA) automated fluorescent microscope facilitated the acquisition of four images of Alexa 488 and Hoechst staining from each well in a culture plate (10× objective,

Nikon Plan Fluor, NA 0.3). The integrated grey value above a user-selected threshold and counts of Hoechst-stained nuclei were quantified using MetaMorph® software (Molecular Devices; v. 6.2r6) and subsequently processed to obtain the average total grey value per cell in an image. Any images affected by brightly fluorescent debris or that were not correctly focused were excluded from subsequent analysis. Data was normalized such that 100% was equivalent to the average of the vehicle control.

GraphPad Prism (v. 4.02, GraphPad Software) and SigmaStat (v. 3.5, Systat Software) were utilized to generate graphs, fit appropriate models and perform statistical tests. Significance was assessed with ANOVA and Tukey post-testing unless only two groups were to be compared, in which case a *t*-test was performed. Repeated measures or paired designs were applied when data for the comparison groups were obtained in parallel. All samples originated from a normal distribution (Kolmogorov–Smirnov test). Groups with significantly different variances (Levene Median test) were assessed with non-parametric tests, as indicated. The equation used to fit a "plateau then exponential decay" model in GraphPad Prism was: $Y = IF\ (X < X_0,\ \text{Plateau}, \text{Bottom} + (\text{Plateau} - \text{Bottom}) \times \exp(-k \times (X - X_0)))$; that is, plateau until $X = X_0$, then exponential decay to Bottom.

2.5. Western blotting

Cell pellets were prepared by washing with PBS, incubating for 2 min with 0.2 g/L EDTA·4Na or trypsin (as appropriate), adding an equal volume of media and centrifuging at 500 × *g* for 5 min. The supernatant was removed and lysis buffer added (150 mM NaCl, 0.5% NonIdet P40, 5 mM EDTA, 50 mM Tris–HCl pH 7.9, protease inhibitor [CØmplete Mini EDTA-free, Roche, Mannheim, Germany]). Samples were incubated on ice for 30 min then centrifuged at 14,000 × *g* for 10 min. For peptide-N-glycosidase F (PNGase) treatment (Sigma-Aldrich), samples were subsequently incubated at 65 °C for 5 min with 52.6 mM β-mercaptoethanol, cooled, and incubated with ~0.04 U enzyme per μg protein at 37 °C for 18 h.

Samples were diluted 1:2 in 2× load buffer (125 mM Tris–HCl pH 6.8, 12% SDS, 40% glycerol, 0.01% bromophenol blue), heated at 37 °C for 30 min, then electrophoresed on 10% Bis-Acrylamide (Bio-Rad, Hercules, CA) 10% SDS Tris–HCl pH 8.8 gels and transferred to Hybond-P PVDF membrane (GE Healthcare, Buckinghamshire, UK). The SeeBlue Plus2 pre-stained standard (Invitrogen) was run alongside samples. Membranes were incubated with 5% non-fat milk (NFM) in tris-buffered saline (TBS) with 0.05% Tween (TBS-T; 30 min at RT), then HA11 antibody diluted 1:5000 in 1% NFM/TBS-T overnight at 4 °C. Membranes were washed (three times, for 10 min with TBS-T) and incubated with horseradish peroxidase-conjugated sheep anti-mouse antibody (AP326P, Millipore, Billerica, MA) diluted 1:2000 in 1% NFM/TBS-T (3 h at RT). Following three further washes, membranes were incubated with ECL-plus (GE Healthcare) for 5 min and the chemiluminescent signal detected with autoradiographic film (GE Healthcare).

3. Results

3.1. CB_1 is present both at the cell surface and in a large intracellular pool in transfected and endogenously-expressing cell lines

The majority of the experiments presented in this study were performed with HEK cells stably expressing HA-tagged rat CB_1. To ensure that the results were not cell type or species specific, experiments central to the findings of this report were also performed on CHO and AtT-20 cells stably expressing HA-tagged human CB_1 and on Neuro-2a cells, a mouse neuroblastoma line that expresses CB_1 endogenously [15,32].

We first characterized the subcellular localization of CB_1 in each of these models. Consistent with previous reports (e.g. [18]), CB_1 staining was detected both at the cell membrane in a continuous and uniform distribution, and intracellularly as a diffuse collection of punctate vesicles which ranged in size and intensity of staining (Fig. 1A). In order to quantify the proportion of CB_1 in the cytoplasm versus at the plasma membrane, CB_1 staining was assessed with and without a brief exposure to trypsin, a proteolytic enzyme. No loss of cells was observed with trypsin treatment in comparison with EDTA-only or PBS-only controls (data not shown). As demonstrated in Fig. 1B, incubation with trypsin rendered surface receptors unrecognizable to the N-terminally directed primary antibodies (green), leaving only intracellular receptor detected (red). CB_1 detected in trypsin-treated cells was divided by total CB_1 to give the proportion of intracellular CB_1 and was thereby determined to account for 25–68% of total expression, depending upon the cell type tested (Fig. 1Ci). The variations in the proportion of intracellular CB_1 were not related in any obvious way to total expression levels in the cell lines tested (Fig. 1Cii).

When lysates from the HEK line were analyzed by Western blot, a prominent species of approximately 64 kDa was detected (Fig. 1Di). Two smaller bands of approximately 50 and 45 kDa were also present, although in much lower abundance. The application of trypsin prior to cell lysis (rendering surface receptors undetectable with anti-HA antibody) resulted in a reduction in signal at the 64 kDa band, however a substantial proportion of receptors retained HA immunoreactivity. This correlated with the approximate amount of intracellular CB_1 observed by immunocytochemistry. No change in the smaller bands was observed, indicating they were likely intracellular protein species and may represent receptor in the synthetic pathway or undergoing degradation. Incubation of both native and trypsinised cell lysate with de-glycosylating enzyme PNGase resulted in the elimination of the 64 kDa band and the majority of anti-HA detected protein appearing at the 50 kDa size (which corresponds with the non-translationally modified predicted size of HA-tagged rCB_1, 53 kDa). This suggests that the majority of intracellular pool CB_1 is full-length and similarly glycosylated to surface CB_1. A lack of detectable signal from wild-type HEK cells not expressing HA-rCB_1 confirmed the specificity of the anti-HA antibody in Western blotting (Fig. 1Dii).

3.2. Surface re-population of CB_1 following agonist stimulation is dependent on the ratio of agonist to inverse-agonist

In agreement with preceding reports (e.g. [16,17]), we observed rapid internalization of CB_1 following the application of agonist. As we have demonstrated previously, endocytosis from the plasma membrane was dependent on the concentration of agonist applied and the duration of stimulation [29]. We sought to investigate the recovery of surface receptors after internalization with cannabinoid agonists HU and WIN. After 15 min of 10 nM HU stimulation (to induce near-maximal internalization), two washes in experimental media at 37 °C and replacement with media containing only vehicle, no re-population of the cell surface was detected over the next five hours (Fig. 2A). However, if the replacement media contained 100 nM SR, a CB_1 inverse-agonist, slow surface re-population was observed (linear regression, $12.0 \pm 1.4\%\ h^{-1}$). Despite an equivalent degree of internalization, the extent of re-population following SR treatment was inhibited at a higher agonist concentration (100 nM; Fig. 2A). Similar trends were observed when internalization was initiated by WIN (data not shown). As shown in Fig. 2B, the extent of receptor re-population following internalization with either agonist exhibited a strong dependence on the concentration of SR applied. These data suggested that several washes with media were insufficient to completely remove either agonist, and that in the absence of a sufficient concentration of SR to compete for binding sites, receptors reaching the cell membrane were rapidly

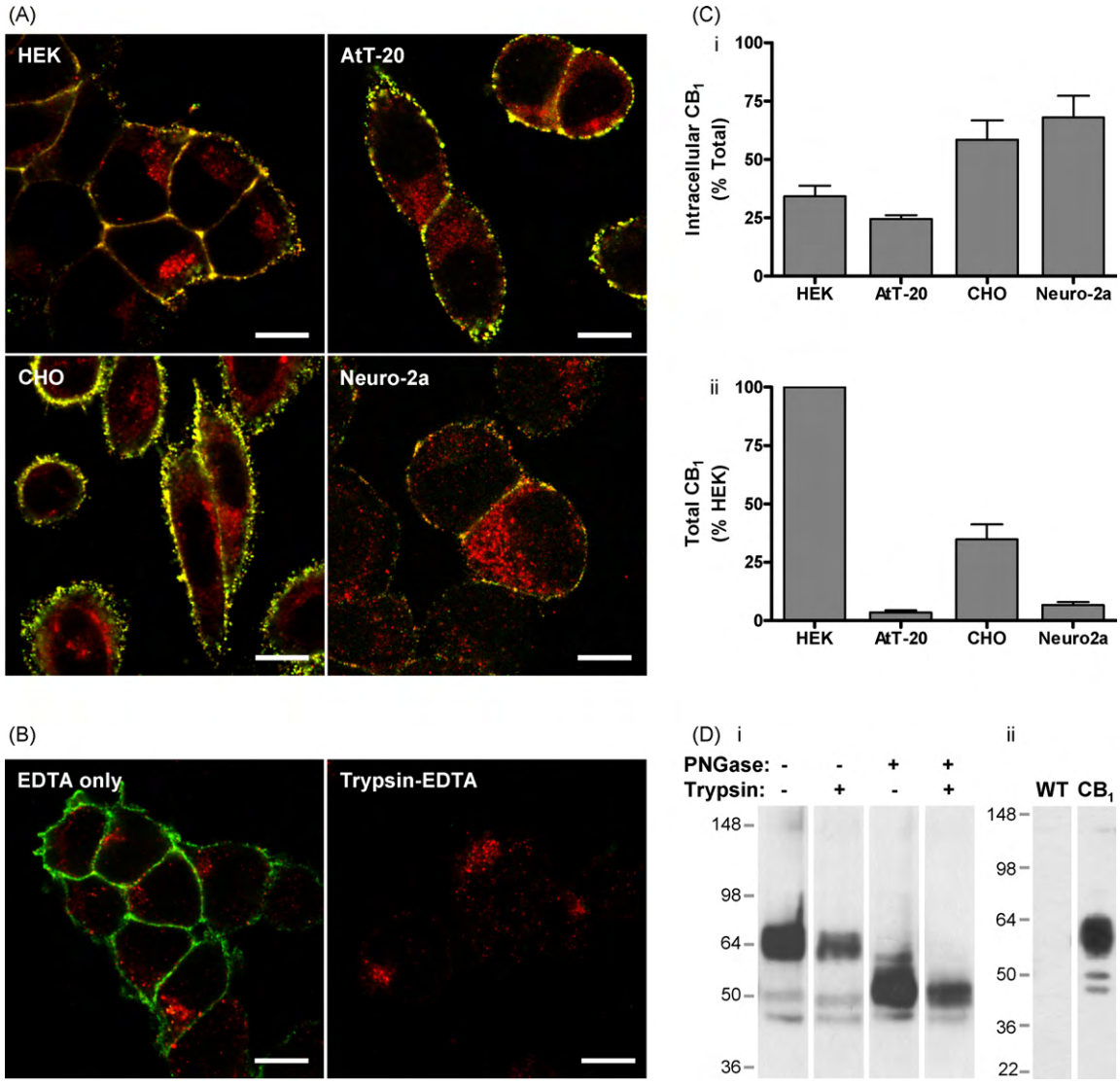

Fig. 1. CB_1 localization in HEK, CHO, AtT-20 and Neuro-2a cells. (A) Confocal micrographs of CB_1 in four cell lines. Live cells were incubated with primary antibody and, following fixation and permeabilization, Alexa Fluor® 488 secondary antibody was applied to detect surface CB_1 (green). Cells were then fixed for a second time and incubated again with primary antibody then Alexa Fluor® 594 secondary antibody to detect total CB_1 (red). (B) Confocal micrographs of rCB_1 HEK cells with and without trypsin treatment. Live cells were incubated with primary antibody, then Alexa Fluor® 488 secondary antibody to detect surface CB_1 (green). Cells were then fixed and permeabilized and incubated again with primary antibody, then Alexa Fluor® 594 secondary antibody to detect intracellular CB_1 (red). (A and B) Bars, 10 μm. Representative of three independent observations. (C) Intracellular CB_1 (trypsin-treated cells) as a percentage of total CB_1 expression (vehicle-treated cells) in the same cell type (i), and total CB_1 as a percentage of HEK rCB_1 total expression (ii). Data are presented as the mean ± S.E.M. of three independent experiments. (D) Western blot for rCB_1 in lysates from HEK cells treated with trypsin and/or PNGase (i). No signal was detected when untransfected wild-type HEK lysate was probed (ii, "WT"). Lanes are from the same film exposure but have been re-arranged for presentation. Protein standard marker in kDa. Representative of three independent observations.

internalized by residual agonist. Consistent with this hypothesis, the surface was also replenished with CB_1 if incubated with 1 μM Con A (an inhibitor of internalization) following agonist-induced internalization (Fig. 2A).

To confirm this hypothesis, the process was visualized by exposing the living cells to primary antibody following agonist stimulation and internalization (Fig. 2C). Immediately after 15 min 10 nM HU stimulation very little receptor was detected, indicating near-complete CB_1 internalization (i). After replacing the media with that containing only vehicle, continued exposure to primary antibody resulted in the detection of a large number of intracellular vesicles but no surface CB_1, consistent with receptors reaching the cell membrane and binding primary antibody, but subsequently internalizing (ii). However, in the presence of a sufficiently competitive concentration of SR, receptors could clearly be detected on the cell surface (iii). If the agonist concentration was increased to 100 nM some re-populating CB_1 was held at the surface. However, a significant proportion was now internalized and appeared in intracellular vesicles (iv).

3.3. *Live antibody labeling indicates internalized CB_1 does not recycle and is instead degraded*

While the assays published previously and thus far described here measured net surface re-population, the results of which have been taken to indicate that CB_1 recycles [17], conclusive demonstration of receptor recycling requires the detection of the same individual receptors that were originally located at the surface returning back to the plasma membrane following internalization. We used a previously established live antibody labeling technique [5] to investigate whether antibody-tagged CB_1 returned to the surface following agonist-induced internalization.

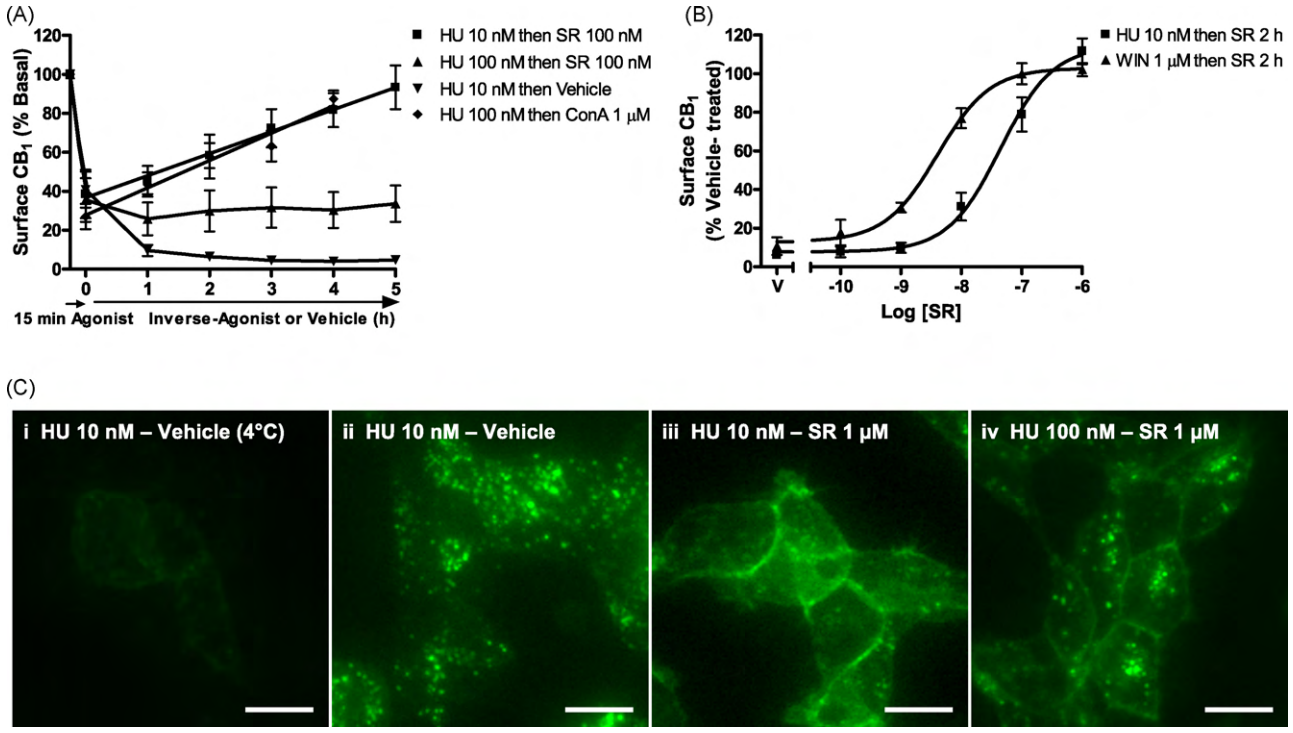

Fig. 2. CB_1 cell surface re-population following agonist-stimulated internalization. (A and B) Surface rCB_1 in HEK cells following 15 min agonist stimulation, washing, and incubation with SR 100 nM, Con A 1 μM, or vehicle for 0–5 h (A), or SR at varying concentrations for 2 h (B). Primary antibody was incubated with live cells at the conclusion of drug stimulation to assay net surface CB_1. Data are presented as the mean ± S.E.M. of four independent experiments. (C) Widefield images of rCB_1 HEK cells treated with agonist at the indicated concentration for 15 min, washed with SFM, then incubated with SR 1 μM or vehicle in the presence of primary antibody for 1 h. The SR or vehicle incubations were at 37 °C, except (i) at 4 °C which demonstrates surface CB_1 immediately following agonist stimulation. In this experiment, only CB_1 delivered to the cell surface subsequent to agonist-induced internalization is labeled with primary antibody. After fixation, secondary antibody was applied under permeabilizing conditions. Bars, 15 μm. Representative of three independent observations.

This technique labels receptors with primary antibody prior to agonist exposure, and we have previously established that this does not alter the rate of internalization [29].

Under conditions where re-population of the surface with CB_1 was observed (internalization with 10 nM HU, followed by incubation with 1 μM SR), no recovery of antibody-tagged CB_1 to the surface was detected for up to six hours (Fig. 3A, "Recycling"). Even in the presence of inverse-agonist, antibody-tagged CB_1 continued to internalize suggesting that the CB_1 that was still resident at the surface at the end of the initial stimulation had been committed to endocytose following interaction with agonist. To ensure that the presence of the primary antibody had not prevented re-population, cells were again exposed to antibody at the end of the experiment and re-population was indeed observed (Fig. 3A, "Re-population"). The CB_1 surface re-population observed with and without the antibody live labeling step at the start of the experiment were not significantly different (two-way ANOVA $P = 0.85$).

To ensure that our method could detect receptor recycling we applied it to HEK cells expressing HA-tagged human D_1, a receptor that has previously been reported to recycle [33]. As shown in Fig. 3B, following antibody labeling, 10 μM dopamine-induced internalization, and washout with D_1 antagonist (SCH 23390 10 μM), recycled antibody-tagged D_1 was clearly detected at the cell surface. It was also notable that the rate of D_1 surface re-population was much more rapid than for CB_1 with the cell surface being essentially re-populated in less than an hour (one-phase exponential association from t_0, $t = 0.20 \pm 0.025$ h, 2.9 ± 0.30 h, respectively; unpaired t-test $P \leq 0.001$).

When antibody-bound receptor was detected with secondary antibody under permeabilizing conditions to label both surface and internalized receptors, total immunoreactivity associated with D_1 was unchanged over the timecourse studied (Friedman ranks one-way ANOVA $P = 0.96$). In contrast, that associated with CB_1 progressively decreased (plateau then one-phase exponential decay, $X_0 = 0.17 \pm 0.076$ h, $t = 1.04 \pm 0.38$ h) until it was equal-to or less than the limit of detection, suggesting that internalized CB_1 had degraded (Fig. 3C). The localization of internalized receptor was also different between the two receptors, with CB_1 internalized to endosomes of varying size and staining intensity that are scattered diffusely in the cytoplasm, whereas internalized D_1 endosomes clustered in a well-defined perinuclear area (Fig. 3D), suggesting that CB_1 enters a different post-endocytic trafficking pathway to D_1.

In analogous experiments to those performed by Martini et al. [28], we repeated the recycling with live antibody labeling experiment using WIN (10 and 100 nM) to internalize both HA-rCB_1 and FLAG-rCB_1 in HEK cells and inverse-agonist AM (5 μM), however again found no evidence of bona fide CB_1 recycling (Fig. 3E and data not shown).

To ensure that the failure of CB_1 to recycle was not unique to HEK cells, live antibody labeling experiments were also carried out on Neuro-2a cells and transfected CHO and AtT-20 cells. In all cell lines the cell surface was re-populated with CB_1 after 10 nM HU-induced internalization and washout with 1 μM SR (Fig. 3F "Re-population"), however no return of antibody-tagged CB_1 to the plasma membrane was detected (Fig. 3F "Recycling").

3.4. Constitutively internalized CB_1 is degraded following endocytosis and does not accumulate to form the intracellular pool

CB_1 is generally considered to be a constitutively active receptor (reviewed in [34]) and ligand-independent constitutive internalization is associated with this behavior [18]. As inferences from previous studies have suggested that the CB_1 intracellular pool is

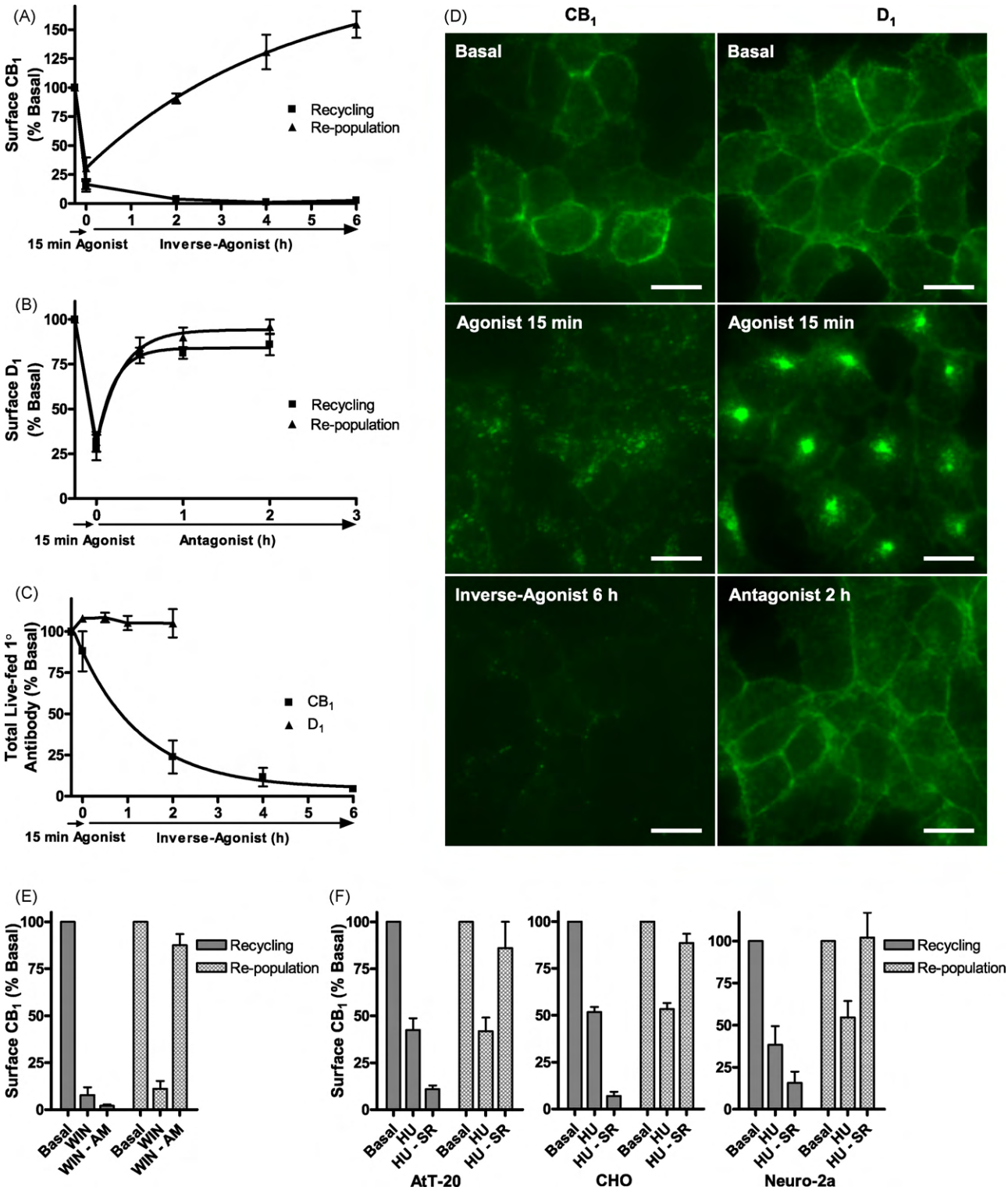

Fig. 3. Antibody live labeling indicates endocytosed CB_1 does not recycle and is instead degraded. (A and B) Surface rCB_1 (A) or hD1 (B) in HEK cells following 15 min agonist stimulation, washing, and incubation with inverse-agonist or antagonist for 0–6 h. Primary antibody was applied prior to agonist stimulation (''Recycling'') or both at the start and at the end of all drug treatment (''Re-population''). Secondary antibody was applied under non-permeabilizing conditions to detect surface primary antibody. (C and D) Cells were treated as in A and B with primary antibody applied prior to agonist stimulation, however secondary antibody was applied under permeabilizing conditions to detect remaining primary antibody in the entire cell. (E) Surface HA-rCB_1 in HEK cells treated as in A with 15 min WIN 100 nM agonist stimulation followed by 5 h AM 5 μM. (F) Surface CB_1 in AtT-20, CHO and Neuro-2a cells treated as in A with 5 h SR incubation. (A–C, E and F) Data are presented as the mean ± S.E.M. of three independent experiments. (D) Bars, 15 μm. Widefield images, representative of three independent observations.

formed as a result of constitutive internalization and contributes to a constitutive recycling pathway, we examined these pathways directly. rCB_1 HEK cells were exposed to primary antibody for 30 min to label surface receptors, washed, and incubated at 37 °C prior to fixation at a range of time points. After applying secondary antibody under non-permeabilizing conditions to detect only primary antibody-bound receptor remaining on the surface, we observed constitutive endocytosis of CB_1 with a half-life of

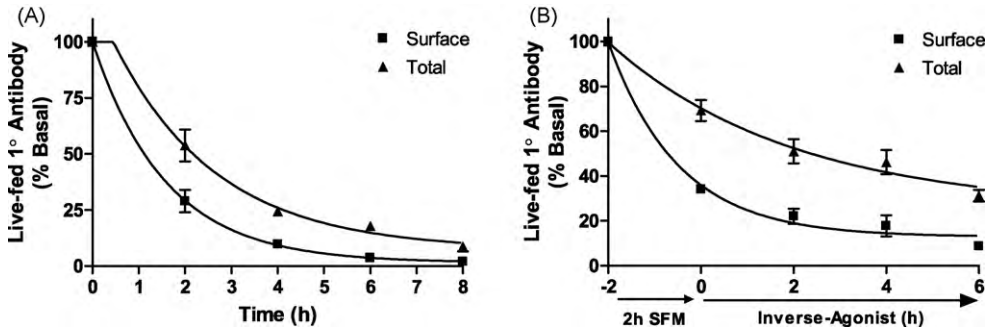

Fig. 4. CB_1 undergoes constitutive endocytosis but is subsequently degraded. (A and B) Surface rCB_1 in HEK cells was labeled with primary antibody and allowed to internalize for 0–8 h (A), or 2 h then exposed to inverse-agonist for 0–6 h to determine whether constitutively internalized CB_1 recycled (B). Secondary antibody was then applied under non-permeabilizing ("Surface") or permeabilizing ("Total") conditions. Data are presented as the mean ± S.E.M. of three independent experiments.

1.29 ± 0.12 h (one-phase exponential decay; Fig. 4A). None of the receptor tagged with antibody at the start of the experiment remained at the surface after 6 h indicating that the entire surface CB_1 population had turned-over. In order to determine the fate of the endocytosed receptors we applied the secondary antibody under permeabilizing conditions and noted a progressive reduction in signal (plateau then one-phase exponential decay, $X_0 = 0.33 \pm 0.29$ h, $t = 1.44 \pm 0.17$ h) indicating that CB_1 was likely degraded following constitutive internalization, rather than recycled to the cell surface or sequestered in the intracellular pool.

To investigate whether the constitutively internalized receptor recycled, we applied antibody to live cells for an extended period (4 h), and then allowed the tagged receptors still at the cell surface at the end of this time to internalize (incubation in vehicle, 2 h) before applying SR 1 μM for 6 h and monitored the antibody-tagged CB_1 for any return to the cell surface and the longevity of signal in the cytoplasm. Again, we did not observe any recovery of antibody-tagged CB_1 to the plasma membrane, and instead both the surface and intracellular signal was progressively lost during the vehicle and SR incubation periods (Fig. 4B).

3.5. Blockade of constitutive internalization results in an up-regulation of surface and total CB_1 which is prevented by protein synthesis inhibition

The interaction of inverse-agonist with CB_1 stabilizes the receptor in an inactive conformation and prevents both constitutive signaling and constitutive endocytosis [34], resulting in the accumulation of receptors at the plasma membrane. Consistent with this model, we have demonstrated that surface CB_1 is up-regulated when cells are incubated with 1 μM SR (one-phase exponential association, $t = 2.74 \pm 0.33$ h, Fig. 5A). Under these conditions, total cellular CB_1 was also increased ($t = 2.82 \pm 0.40$ h), indicating that at least a proportion of the receptors stabilized at the surface derive from the synthetic pathway. A similar rate of SR-induced up-regulation was observed when cells were incubated with Con A ($P = 0.54$), while treatment with monensin (which inhibits vesicle acidification and thereby trafficking to the cell surface [35]) inhibited surface up-regulation (data not shown). As well as changes in surface and total receptors, we also directly monitored the size of the intracellular pool by performing permeabilizing immunocytochemistry on cells briefly treated with trypsin (as described above). We did not observe any significant change in the size of the intracellular pool following incubation for 5 h with SR (two-way ANOVA $P = 0.66$), despite there being significant increases in both cell surface and total receptor expression (both $P = 0.0005$; Fig. 5B, left panel). Surface and intracellular receptor expression were measured independently (live antibody labeling or permeabilized labeling of trypsin-treated cells, respectively). Supporting the validity of these measurements, when summed ("Surface + Intracellular") equivalent results to the directly measured total expression level ("Total") were obtained. Similar results were obtained with endogenous CB_1 in Neuro-2a cells (Fig. 5B, right panel).

The lack of change in intracellular pool size suggests that surface CB_1 up-regulation is due to the delivery of newly synthesized receptors rather than mobilization of the intracellular pool. This hypothesis was further investigated by applying a protein synthesis inhibitor, CHX. CHX (10 μg/mL) completely inhibited surface and total CB_1 up-regulation following SR treatment (Fig. 5C). This finding was replicated in our three other cell models at a single time point (Fig. 5D). After 6 h at this CHX concentration cells appeared morphologically normal and cell density was not markedly reduced (data not shown). We repeated this experiment by Western blot with HEK cells, comparing total CB_1 with that still detectable in trypsin-treated cells (i.e. intracellular pool only) and again observed no change in the size of the intracellular pool with SR treatment. However, the intracellular pool-associated signal decreased with CHX treatment regardless of the presence of SR (Fig. 5E). CHX also completely prevented the re-population of surface CB_1 following agonist-induced internalization in HEK cells (data not shown).

3.6. Surface, but not intracellular CB_1, is degraded with chronic agonist stimulation

A common consequence of chronic GPCR stimulation is overall receptor downregulation. We demonstrate that this is indeed the case for CB_1, and the extent and timecourse of downregulation are dependent on the concentration of agonist applied (Fig. 6A and B). The maximum half-life of degradation observed was 0.82 ± 0.29 h with 1 μM HU (one-phase exponential decay; Fig. 6A). Interestingly, the chronic agonist effect reached a plateau at 55.5 ± 2.5% of the starting expression level. We observed similar results with WIN (Fig. 6B) and with our other cell models (data not shown).

We were not able to induce any further degradation by incubating with agonist for longer time periods (up to 48 h) or by replenishing the incubation media with fresh agonist (data not shown). We also investigated whether up-regulating surface receptors with SR prior to the addition of chronic agonist would alter the extent of degradation. However the amount of receptor remaining following chronic agonist treatment did not change (data not shown), suggesting that the influx of receptors to the cytoplasm upon agonist stimulation was not saturating the degradation pathway. This was confirmed with a live antibody labeling experiment, whereby the fate of surface receptors labeled with primary antibody prior to agonist stimulation was observed. Indeed, the antibody–receptor signal was lost well within the 6 h time period studied (data not shown). To ensure that this did not represent that only a sub-population of cells were responding to

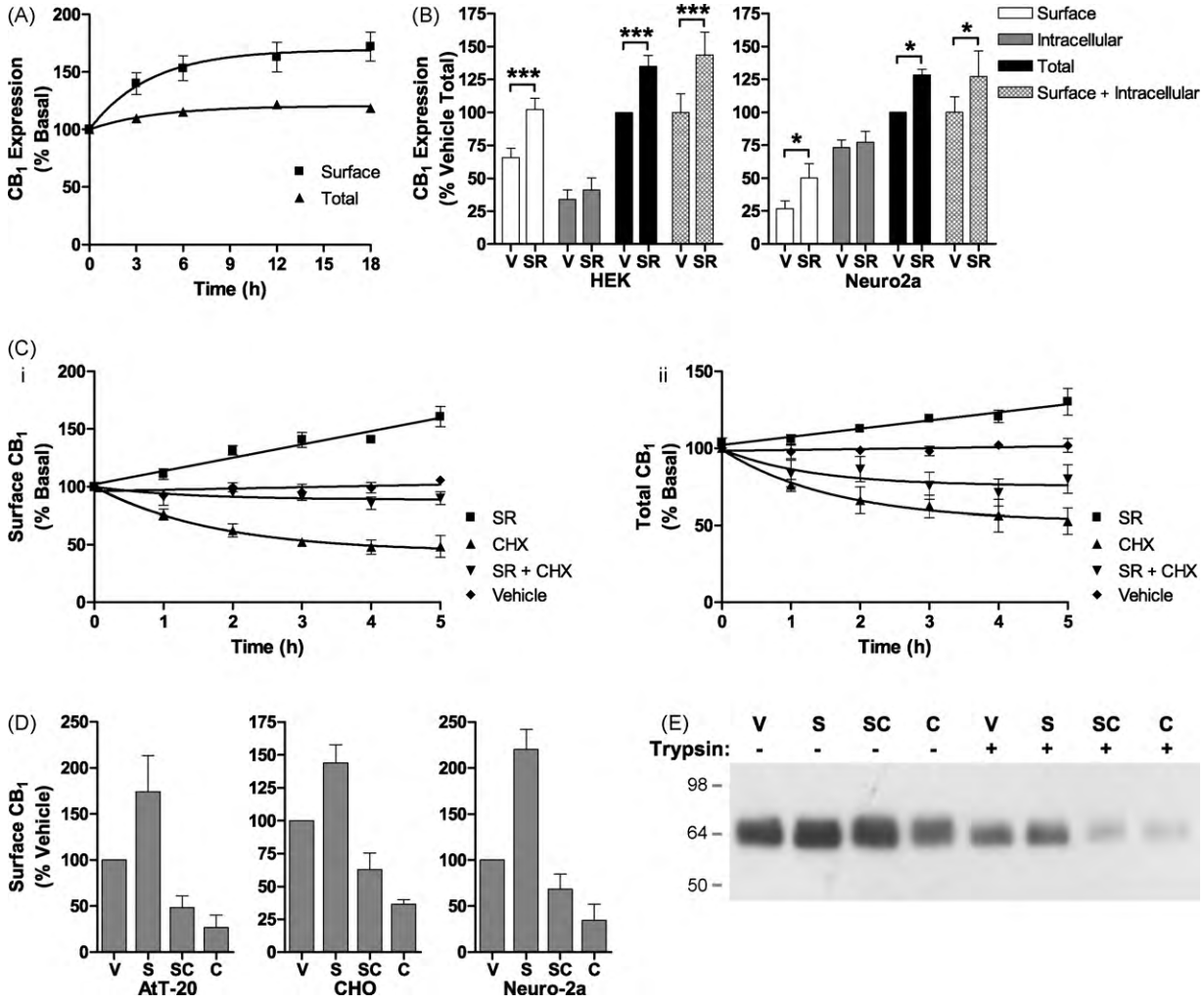

Fig. 5. Inverse-agonist induced cell surface up-regulation is blocked by protein synthesis inhibition. (A) Surface and total rCB_1 expression in HEK cells following 0–18 h incubation with 1 μM SR. (B) Surface, intracellular, and total CB_1 expression in HEK and Neuro-2a cells following incubation with vehicle or SR for 5 h. Data are normalized to total CB_1 expression in vehicle-treated cells. "Surface + Intracellular" represents the sum of independently obtained surface and intracellular expression levels (live antibody labeling or permeabilized labeling on trypsin-treated cells, respectively), whereas "Total" is the total expression level obtained by permeabilized antibody labeling on cells that were not trypsin treated (EDTA only). (C) Surface (i) or Total (ii) CB_1 in HEK cells following 0–5 h incubation with SR and/or CHX. (D) Surface CB_1 in AtT-20, CHO and Neuro-2a cells following 5 h incubation with SR and/or CHX. (A–D) Net surface receptor was assayed by incubating live cells with primary antibody at the conclusion of drug stimulation. Data are presented as the mean ± S.E.M. of three independent experiments (*, $P < 0.05$; ***, $P < 0.001$). (E) Western blot of CB_1 in lysates from HEK cells treated with SR and/or CHX for 6 h. Samples not exposed to trypsin demonstrate total CB_1, while those treated with trypsin reveal intracellular CB_1. Protein standard marker in kDa. Representative of three independent observations.

the agonist stimulation, we performed single-cell analysis on cell populations treated for 0–6 h with agonist and found that the distribution of cellular responses was uni-modal with no evidence of multiple populations of cells that might be responding differentially to the treatment (data not shown).

3.7. Intracellular CB_1 does not co-localize with Gα subunits

The experiments described herein demonstrate that the localization of intracellular CB_1 is not influenced by agonist or inverse-agonist stimulation. However, a recent study indicated

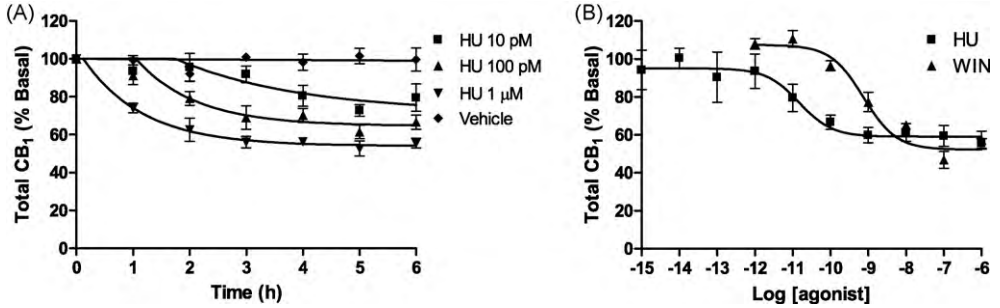

Fig. 6. Chronic stimulation with agonist results in CB_1 degradation. (A and B) Total rCB_1 in HEK cells treated for 0–6 h with HU at three concentrations or vehicle (A), or for 6 h with varying concentrations of HU or WIN (B). Data are presented as the mean ± S.E.M. of three to four independent experiments.

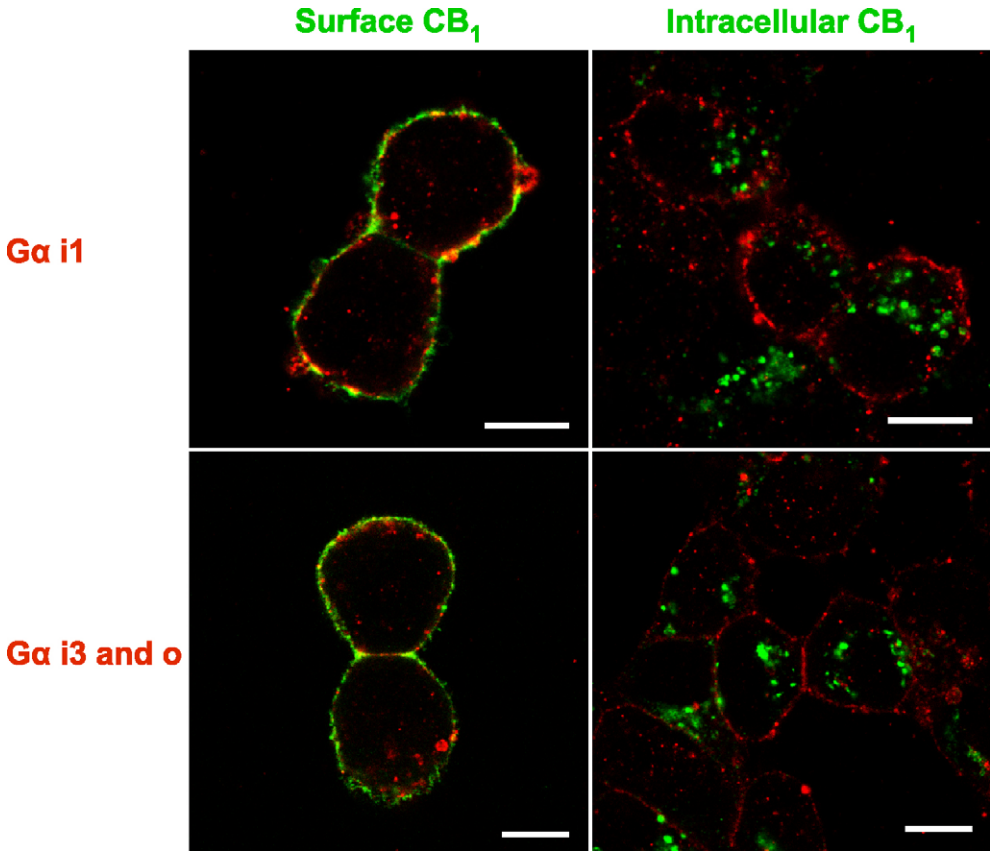

Fig. 7. Intracellular CB_1 is not co-localized with inhibitory Gα subunits. Confocal micrographs of rCB_1 HEK cells stained for surface or intracellular CB_1 (green) and Gα i1 or i3 and o (red). Bars, 10 μm. Representative of three independent observations.

that intracellular CB_1 may be responsible for agonist-mediated signaling responses [23]. We were therefore interested to determine whether Gα subtypes known to mediate CB_1 signaling [36] co-localized with the CB_1 intracellular pool. HEK cells probed with antibodies to Gα i1, or i3 and o, demonstrated predominantly surface staining (red, Fig. 7), and Gα labeling co-localized with surface CB_1 (detected by incubating primary antibody with live cells prior to fixation; green). Although a small amount of cytoplasmic staining was noted, we did not observe co-localization of any of these G-protein subunits with the CB_1 intracellular pool (detected by incubating primary antibody under permeabilizing conditions on trypsin-treated cells; green).

4. Discussion

As a highly prevalent receptor that modulates a range of brain and systemic functions, CB_1 is currently of significant interest as a pharmaceutical target. The number of functional receptors present at the plasma membrane is generally considered to be a major determinant of potential cell responsiveness to endogenous or exogenous ligands, yet we and others have noted that many cells that endogenously express CB_1, including neurons, exhibit large intracellular pools of receptors in addition to those at the plasma membrane (e.g. [9,14,15]). This is also the case in cells with introduced CB_1 (e.g. [16,17]). One estimate determined that only ~15% of the receptors appear on the cell surface regardless of total receptor number [18]. These consistent observations have lead to the hypothesis that the CB_1 intracellular pool represents a reservoir of receptors that the cell can utilize to exhibit highly dynamic responses to physiological and pathological stimuli [16,18]. Consistent with this theory, intracellular CB_1 does not co-localize with cellular machinery responsible for new receptor synthesis [18]. Previous studies have established that CB_1 receptors undergo rapid internalization following activation [17], however the processes that control its post-endocytic fate are not yet well established. Although it has been suggested that the CB_1 can recycle constitutively [18] or following agonist stimulation [17,28], efficient degradation has also been reported [17,19,28], as has co-localization of intracellular CB_1 with markers of the degradative pathway [23,28].

This study has utilized both cells expressing endogenous CB_1, and a range of cell lines transfected with CB_1 to investigate these processes further. In the cell lines studied approximately 46% (±10%) of CB_1-associated staining was present intracellularly in vesicles of varying size. The relative size of the intracellular pool varied somewhat between cell types but was independent of overall expression level (see also [18] for a rigorous investigation of this hypothesis). We utilized proteolytic cleavage of the CB_1 extracellular domain to examine the intracellular receptor by Western blotting. This approach revealed that the predominant CB_1-associated bands for total and intracellular CB_1 were of the same size and similarly glycosylated, indicating that the majority of receptors in the cell had been post-translationally modified. Thus, a lack of glycosylation was not responsible for inefficient delivery of CB_1 to the cell surface, as has been noted to occur for other receptors (e.g. [37]).

To investigate post-endocytic trafficking, we initially applied agonist to stimulate internalization and observed the subsequent recovery of surface CB_1. The plasma membrane could indeed be re-populated with CB_1. However, the extent was crucially dependent on the concentration of inverse-agonist applied and less re-population was observed as the agonist concentration was increased. Although it might be anticipated that the application of higher concentrations of agonist could re-route CB_1 from a recycling to a degradative pathway (as suggested in [28]), we

demonstrate that surface re-population occurred so long as the concentration of SR applied was sufficiently high to compete off agonist. This phenomenon cannot be explained by SR's potential for interaction with intracellular receptors, as cell surface re-population also occurred when Con A (which prevents receptor internalization) was applied, suggesting that a net accumulation of CB_1 at the cell surface was facilitated simply by the prevention of further internalization. Furthermore, live antibody labeling experiments indicated that CB_1 was constitutively delivered to the cell surface regardless of the presence of inverse-agonist but was re-internalized unless sufficient inverse-agonist was present. Importantly, these results indicate that simply exchanging culture media is not sufficient to washout cannabinoid agonists HU and WIN, and that subsequent agonist-induced internalization must be prevented in order for CB_1 to accumulate at the cell surface. This might be predicted from the lipophilic nature of cannabinoids resulting in a propensity to remain in the hydrophobic cell membrane and not be released into the aqueous assay media, even when a concentration gradient is present. This inability to washout agonist has important implications for the design of future experiments, and interpretation of previous studies. Therapeutically, this also suggests that multiple applications of low concentrations of cannabinoids may result in the accumulation of ligand in the cell membrane and higher effective concentrations being available to the receptor than expected. This is particularly relevant in the case of cannabinoids, some of which are known to exhibit long half-lives *in vivo* (e.g. Δ^9-THC) and can take several weeks to be completely eliminated (reviewed in [38]). Furthermore, drugs with different lipophilicity profiles may produce different effects *in vivo* despite exhibiting similar pharmacological characteristics *in vitro*, as has been reported for other lipophilic GPCR ligands such as β-adrenergic receptor antagonists (reviewed in [39]).

In order to investigate bona fide receptor recycling, that is, the return of the same receptor units to the cell surface following internalization, we applied a live antibody labeling technique which has been utilized to demonstrate receptor recycling previously [5]. While robust recycling of the D_1 receptor was observed under these conditions, recycling of antibody-tagged CB_1 was not detected in any of our cell lines. This was not due to the presence of the primary antibody inhibiting recycling, as surface re-population was not influenced by the live antibody labeling performed at the start of the experiment. This finding is in contrast to that of Martini et al. [28] who observed recovery of M1 anti-FLAG antibody-tagged CB_1 to the surface following stimulation with WIN in HEK cells. However, their model system utilized a N-terminal signal sequence which might have influenced the trafficking of their receptors: signal sequences typically result in excessive receptor over-expression (e.g. [40]) which may have lead to the re-direction of some receptors to abnormal pathways. The recycling of D_1 to the cell surface was much faster than the re-population of the cell surface with CB_1, consistent with a different mechanism of receptor delivery. Furthermore, internalized D_1 and CB_1 receptors displayed notably different distributions with internalized D_1 congregating in a localized area of the cytoplasm but CB_1 being dispersed throughout the cytoplasm. Finally, while the total immunoreactivity associated with internalized D_1 was unchanged over the timecourse studied, that associated with internalized CB_1 was lost, suggesting that CB_1 was degraded. If CB_1 utilizes a different recycling pathway to D_1 there might be opportunity for the antibody to be cleaved or dissociate from CB_1, resulting in a false-negative, however the rate of antibody/receptor degradation observed in this experiment was not significantly different to that for the overall agonist-induced degradation rate when monitoring total CB_1 ($P = 0.78$). This suggests that, once bound, the antibody lifespan mimics that of the receptor. "Slow" and "fast" recycling routes have been described for GPCRs [41], with the return of surface expression to basal levels typically occurring within 60 min (e.g. [42]) and 3 h (e.g. [43,44]), respectively. Near-complete recovery of surface thrombin receptors resulting from intracellular pool mobilization has also been observed within 60 min [45]. While the rate of CB_1 surface re-population is similar to that of recycling via the "slow" route, this pathway is independent of protein synthesis [43], whereas we demonstrate that CB_1 surface re-population is abolished in the presence of CHX.

The ability of inverse-agonist to induce surface CB_1 up-regulation has been demonstrated previously and was assumed at the time to represent the stabilization of constitutive recycling receptors from the intracellular pool to the surface [16,18]. This assumption was supported by the observation that monensin inhibited this up-regulation [18], as replicated in this study. However, as an inhibitor of vesicle acidification, monensin not only blocks recycling vesicles but also vesicles transporting newly synthesized cargo from the Golgi apparatus [35]. Our evidence instead suggests that inverse-agonist induced surface up-regulation is a result of newly synthesized receptors being stabilized at the cell surface and therefore protected from constitutive degradation. Accordingly, an up-regulation of total receptor number was also observed. Although this mechanism would not preclude concurrent recycling or mobilization, we present two crucial lines of evidence to strongly support our hypothesis. Firstly, inclusion of a protein synthesis inhibitor in the assay medium completely abolished the surface up-regulation, and secondly, the size of the intracellular pool did not change with inverse-agonist incubation as would be expected if CB_1 was being mobilized from the intracellular pool. The latter finding contradicts that reported by Leterrier et al. [18], in which the size of the intracellular pool appeared to decrease with SR incubation and no increase in total receptor-associated fluorescence was observed. However, as that study utilized transient transfection and manual quantification of just a few individual cells, subtle changes in total expression may have been difficult to determine. Furthermore, transient transfection of receptors has been reported to alter aspects of the cell cycle and induce apoptosis [46] which could in-turn influence receptor signaling and trafficking. One prior study has disputed the ability of inverse-agonist to prevent constitutive internalization, however this is in contrast to a number of other reports (see above) and the N-terminally chimerized GFP tag utilized might have influenced inverse-agonist binding or activity [13].

Inverse-agonist up-regulation and surface re-population following agonist stimulation occurred at rates that were not significantly different ($P = 0.72$) and both were sensitive to CHX, indicating that these assays likely represented the same pathway of receptor delivery to the surface. We cannot, however, exclude the remote possibility that CHX exerted its effects by preventing the production of a protein essential for CB_1 recycling or intracellular pool mobilization.

While both surface re-population and up-regulation appear to represent the stabilization of newly synthesized receptors at the surface, the possibility remained that constitutive cycling between the cell surface and intracellular pool might exist at a low level and therefore not be detected in our previous assays. Putative constitutive trafficking was determined by the rate of internalization in the absence of exogenously applied agonist. When measuring constitutive internalization with live antibody labeling, instead of the maintenance of antibody-bound receptor in the cytoplasm as would be expected if constitutively internalized receptors were joining the intracellular pool, CB_1-associated antibody was degraded. This degradation occurred over a time-course that was not significantly different to that observed for overall receptor turnover (as measured by incubation with CHX; $P = 0.26$). The constitutive internalization rate also correlated well

with that of cell surface downregulation with CHX treatment ($P = 0.51$) indicating that in order to maintain a steady state, constitutively internalized receptors were replaced by newly synthesized receptors. Our results therefore suggest that, while at any one time a small proportion of intracellular receptors may have recently constitutively internalized (and be en-route to degradative organelles), the remaining cytoplasmic receptors residing in the intracellular pool have not, and will not, reach the cell surface in their lifetime.

Downregulation of GPCRs with chronic agonist stimulation is a commonly noted occurrence [47], and indeed this phenomenon has been observed for CB_1 both *in vitro* (e.g. [19,28]) and *in vivo* (reviewed in [27]). In all cell lines studied, the application of agonist resulted in CB_1 degradation over a matter of hours and the effect was agonist concentration-dependent. However even with the highest agonist concentrations tested and long or multiple stimulations (to compensate for potential ligand instability), ~50% of CB_1 remained and was not degraded (compared to vehicle-treated cells). This parallels a number of *in vivo* studies, whereby chronic agonist treatment reduced CB_1 binding by up to 50% (dependent on brain region; reviewed in [48]), or CB_1 protein by up to 65% [49]. As we confirmed the degradative pathways were not being saturated by newly endocytosed CB_1, it appears that this residual CB_1 represents the intracellular pool which does not undergo trafficking in response to agonist treatment. The only condition under which a greater proportion of cellular CB_1 was degraded was when incubated with CHX (which trended towards the eventual loss of all CB_1), indicating that although unresponsive to agonist and inverse-agonist, receptors in the intracellular pool do undergo turnover.

The body of evidence described so far strongly suggests that the receptors in the intracellular pool are unresponsive to agonist or inverse-agonist stimulation. However, a recent study has suggested that the intracellular pool represents the predominant site for CB_1-mediated signaling [23]. Although we have not addressed this question directly, we note that Gα subtypes i1, i3 and o are predominantly localized at the plasma membrane and do not co-localize with intracellular CB_1. It therefore seems unlikely that intracellular CB_1 could be signaling via archetypal G-protein pathways, although this does not discount the possibility that intracellular CB_1 might signal via non-G-protein-mediated mechanisms. While Rozenfeld and Devi demonstrate a small amount of CB_1-$G\alpha_i$ co-immunoprecipitation, interaction of intracellular CB_1 with $G\alpha_i$ may occur in the synthetic pathway [50]. As no surface CB_1 was detected by Rozenfeld and Devi [23] in Neuro-2a cells, it may be that their culture had an unusual phenotype which might also contribute to these incongruous findings. The architecture of the majority of cannabinoid neuronal networks in the brain reduces the likelihood that activation of intracellular CB_1 occurs *in vivo* because eCBs are typically released from activated dendrites in order to act at pre-synaptic CB_1, although activation of somatic CB_1 may play a role in some processes such as somatodendritic slow self-inhibition [51].

While the results of this study are consistent between four cell models, one of which expresses CB_1 endogenously, it will be important to confirm and further investigate these findings in more complex model systems. It is certainly possible that different cell populations express diverse complements of trafficking adaptor proteins *in vivo* which in-turn may influence CB_1 trafficking, and the identification and characterization of CB_1 interacting proteins is an exciting area of current research. One quality of CB_1 trafficking in hippocampal primary culture neurons that has now been reported by two groups, but that we could not replicate in our cell lines, was that of constitutively internalized CB_1 being trafficked (and therefore recycled) to the axonal plasma membrane [12,13]. Indeed, it may be anticipated that undifferentiated cell lines may lack adaptor proteins crucial for axonal transport and targeting, or that even if these proteins were expressed, the cargo may be degraded due to the absence of the target cellular domain. However both of these studies utilized chimerization of a large fluorescent tag which may have artefactually influenced the results. In addition, it should be noted that studies on cultured primary neurons are not without limitations; these cells exhibit considerable CB_1 expression in dendrites and on the surface along axonal projections which does not match the expression pattern typically observed in detailed ultra-structural studies on brain sections (e.g. [9,52,53]). CB_1 is also expressed on some non-polar cell types such as microglia [54] and peripheral immune cells [55], which our cell lines may well model very closely.

It is interesting to note that CB_1 is one of the most abundant GPCRs in the brain and a significant proportion of that receptor population resides intracellularly in the cell soma [9,21,22]. While it was previously inferred that this intracellular pool represented potentially functional receptors that may be mobilized to the cell surface and contribute to cannabinoid responsiveness [18], the data presented in this study suggest that these receptors cannot be mobilized to the surface. This also implies that aside from its important roles in brain function, high levels of CB_1 in the nervous system may be partly due to compensatory mechanisms for the significant degree of inefficiency in CB_1 delivery to axon terminals. The production of a large number of receptors, only to reside in an intracellular pool with no apparent function, seems to be an extremely wasteful application of cellular energy. The inefficient translocation and subsequent processing of CB_1 in the endoplasmic reticulum (ER) has been described previously and was correlated with CB_1s long extracellular tail and lack of endogenous signal sequence [40]. Similar inefficiencies in GPCR production have been reported for other receptors whereby immature receptor is retained in the ER and subsequently degraded (e.g. [56]). However, the intracellular receptors in our study are fully glycosylated and the intracellular pool is only marginally co-localized with ER and Golgi markers. It would therefore appear that the majority of the intracellular pool receptors have traversed the synthetic pathway successfully and a different mechanism must be at play. We also note that when a preprolactin signal sequence is chimerized to CB_1 and expressed in HEK cells, the intracellular pool is still present in similar proportions to cells expressing the wild-type receptor construct (data not shown); although, in line with findings of Andersson et al. [40], the overall expression level is enhanced. It is also unlikely that the formation of CB_1's intracellular pool might be a result of saturation of an adaptor protein, or conversely high levels of CB_1 expression, as the presence and size of the pool is independent of expression level (this study and [18]).

Other post-translational modifications which might differentiate intracellular from surface CB_1 include phosphorylation, ubiquitination and palmitoylation; all of which have been correlated with differential targeting or fate of GPCRs previously (e.g. [57]). Pharmacological chaperones have also been described which can increase the efficiency of GPCR folding and maturation and/or stability following synthesis [58]. If the CB_1 intracellular pool receptors are indeed mature and potentially functional, these approaches could be exploited to enable completed processing and/or re-routing of these receptors thereby enhancing cellular cannabinoid sensitivity.

In summary, our results indicate that constitutive and post-stimulatory delivery of CB_1 to the cell surface is as a result of new receptor synthesis, not recycling or intracellular pool mobilization. Whether as a result of constitutive activation or agonist stimulation, endocytosed CB_1 appears to be degraded and does not contribute to the formation of the intracellular pool. Our data suggest that this pool is instead a reservoir of receptors not delivered to the cell surface and awaiting degradation. These

receptors do not re-distribute upon agonist or inverse-agonist stimulation, although they do undergo turnover. The importance of receptor intracellular trafficking in short- and long-term drug efficacy and the development of drug tolerance implies that these novel findings may have a significant impact on the design and application of cannabinoid therapeutics. These results also suggest that important areas for further investigation include the regulation of CB_1 synthesis, targeting of newly synthesized CB_1 to particular cellular domains, the potential for mobilization of the intracellular pool and modulation of CB_1 targeting to the degradation pathway.

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