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INTRACELLULAR TRAFFICKING OF CANNABINOIDS
RECEPTOR 1

A THOROUGH CHARACTERISATION AND INVESTIGATION INTO THE ROLE OF THE INTRACELLULAR POOL

NATASHA LILLIA GRIMSEY

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmacology, The University of Auckland, 2010
ABSTRACT

Cannabinoid Receptor 1 (CB₁), an abundant G-protein coupled receptor (GPCR) in the central nervous system, is currently of significant interest as a therapeutic target. The cellular control of receptor trafficking is intimately linked with drug effects, however in comparison with other GPCRs, the study of CB₁ trafficking is in its infancy. Although the existing literature suggests CB₁ should be classified as a “dual-fate” receptor, some conflicting evidence exists as to the conditions under which CB₁ recycles or degrades. Of particular interest is the widely noted intracellular pool which has been speculated to form part of a constitutive internalisation and recycling pathway.

This study performs a detailed quantification of CB₁ trafficking in four cell lines, one of which expresses CB₁ endogenously. A novel high-throughput immunocytochemistry-based approach is applied to quantitatively measure receptor trafficking. An important advance on previous studies is the use of a proteolytic method to directly quantitate intracellular receptors. Contrary to previous reports, the data suggests that CB₁ does not recycle following constitutive or agonist-induced internalisation but instead exhibits a primarily degradative phenotype. Evidence is obtained through antibody “live-feeding” protocols and the effects of protein synthesis inhibitors, among other approaches. In addition, the data suggests that the intracellular pool does not traffic to the cell surface and therefore does not contribute to CB₁ signalling via classical paradigms. The effects of Rab GTPase dominantly-acting positive and negative mutants on basal CB₁ localisation corroborate these results.

The findings of this thesis have significant implications for the interpretation of CB₁ biochemical studies and call for a revision of the currently held theories of CB₁ intracellular trafficking. The study provides a foundation for further mechanistic studies and may impact the design and application of cannabinoid therapeutics.
ACKNOWLEDGEMENTS

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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>β2AR</td>
<td>β2-adrenergic receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CB1</td>
<td>Cannabinoid receptor 1</td>
</tr>
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<td>CB2</td>
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<td>cycloheximide</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>D1</td>
<td>Dopamine receptor 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>EC50</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FSM</td>
<td>full-serum media</td>
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<td>G-protein</td>
<td>GTP binding protein</td>
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<tr>
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<td>GPCR-associated sorting protein</td>
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<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>HA</td>
<td>haemagglutinin</td>
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<td>human embryonic kidney-293</td>
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<tr>
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<td>PNGase F</td>
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<td>RM</td>
<td>repeated measures</td>
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<td>SR 141716A</td>
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<td>t½</td>
<td>half-life</td>
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<tr>
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<td>Tween</td>
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<td>trypsin inhibitor from soybean</td>
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<td>wild-type</td>
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CHAPTER ONE

INTRODUCTION

The psychoactive and therapeutic properties of cannabis have been recognised for thousands of years, however it has only been in recent decades that our understanding of the molecular basis of cannabinoid action has advanced significantly. The cannabinoid system is now known to be an integral mediator of a range of normal physiological processes and is a prime therapeutic target for a range of conditions from obesity to neurodegenerative disease. However, much remains to be learnt about endogenous cannabinoid function, its roles in disease pathology, and the effects of exogenous manipulation.

Cannabinoid Receptor 1 (CB₁), the first mediator of the effects of cannabis to be identified (Matsuda et al., 1990; Gerard et al., 1991), is one of the most abundant G-protein coupled receptors (GPCRs) in the mammalian central nervous system and is also expressed at various sites in the periphery (Fride and Gobshtis, 2007). As an important modulator of a variety of brain and systemic functions that is implicated in a number of neurological and immune disorders, CB₁ is the target of a number of therapeutics recently approved or currently in clinical trials (eg. Dronabinol, Nabilone, Marinol, Sativex; reviewed in Maccarrone et al., 2007).

Receptor trafficking between intracellular compartments is an important and popular area of current research. Cellular regulation of surface receptor expression via synthesis, endocytosis, recycling and downregulation has multiple roles in GPCR signalling and regulation (reviewed in von Zastrow, 2003). In particular, these pathways are intrinsically linked to drug sensitivity and
tolerance due to their influence on receptor availability, and consequently the ability of agonists to generate an effective response (Ferguson, 2001). While a number of studies have addressed CB₁ intracellular trafficking, the field is in its infancy in comparison with many other GPCRs. An improved understanding of basal and ligand-mediated CB₁ trafficking will assist in understanding how existing cannabinoid drugs work, the design of new therapeutic approaches and perhaps provide further insight into disease caused by dysfunction of the endocannabinoid system.

This introduction will summarise GPCR intracellular trafficking and review the current state of the literature regarding CB₁ function, pharmacology, and trafficking.

**G-Protein Coupled Receptor Intracellular Trafficking**

GPCRs comprise a large super-family of heptahelical transmembrane-spanning receptors that facilitate numerous wide-ranging physiologies and represent important therapeutic targets (reviewed in Pierce et al., 2002). Signal transduction is generally initiated by ligand interaction with cell surface-resident receptors. Agonist binding stabilises receptor in a conformation that activates intracellular heterotrimeric GTP binding proteins (G-proteins) and consequently intracellular signalling cascades are initiated. The G-protein heterotrimer consists of an α subunit and a βγ dimer, specific subtypes of which are activated by particular GPCRs or ligand-induced conformations and confer differential downstream effects (reviewed in McCudden et al., 2005).

Shortly following receptor activation, the responsiveness of cells to continued stimulation with receptor ligand is commonly observed to wane significantly in a process referred to as desensitisation. A rapid mechanism via which this “switching off” occurs is through receptor phosphorylation by GPCR kinases (GRKs) and binding of an arrestin family member which
sterically hinders interaction of G-protein with the receptor. Receptors must be dephosphorylated in order to regain signalling functionality. This does not occur at the plasma membrane. Instead, receptors are endocytosed into cytoplasmic vesicles where dephosphorylation takes place (reviewed in Lefkowitz, 1998; Moore et al., 2007). Following endocytosis, the cell must direct receptors to appropriate fates within the cell. A crucial decision is between the targeting of receptors back to the cell surface, resulting in resensitisation of the cell to the receptor ligand (“recycling”) versus targeting of receptors to a degradation pathway, resulting in downregulation of the receptor and inhibition of resensitisation (“degradation”). The following section describes these trafficking pathways. A number of differences exist between the trafficking characteristics for different receptor types and the mechanisms via which these are controlled. As it is not possible here to review the entire field of GPCR trafficking in detail, the most common and well-characterised pathways will be explored primarily.

**Intracellular trafficking pathways**

**Internalisation and early endosomal sorting**

As described briefly above, following ligand-induced or constitutive activation (as a result of spontaneous conformational switching), most GPCRs undergo internalisation from the cell surface. This process of sequestration is usually initiated by non-visual arrestin (β-arrestin 1 or 2) binding and the association of receptor with clathrin-coated pits or caveolae. Assembly of triskelion-shaped clathrin and adaptor protein-2 or members of the caveolin protein family produce membrane invaginations that incorporate the once-activated receptor and eventually separate from the plasma membrane in a scission event catalysed by large GTPase dynamin and its binding partners (Hill et al., 2001; reviewed in Claing et al., 2002). For at least some receptor types, internalisation via clathrin-coated pits versus caveolae may be an early indicator of receptor fate (Di Guglielmo et al., 2003). A minor population of GPCRs may also internalise via clathrin/caveolae-independent pathways (Raposo et al., 1989; Moore et al., 2007).
Endocytosis is generally observed to occur rapidly (in the order of minutes), and although not dependent on G-protein signalling, the rate and extent is associated with ligand affinity and efficacy for exerting signalling responses (eg. Turner et al., 2001). A notable exception to this is the opioid receptors which undergo internalisation following activation with most opioid agonists, but not the highly potent and efficacious agonist morphine. Morphine tends to induce marked tolerance in comparison to other opioid agonists. This effect is linked with the lack of receptor internalisation, and consequential de-phosphorylation and resensitisation (Keith et al., 1996).

Newly formed receptor-containing vesicles subsequently proceed to fuse with mildly acidic early endosomes. It is at this stage that de-phosphorylation by G-protein receptor phosphatase (GRP) takes place and ligand dissociation generally occurs (Mellman, 1992; Krueger et al., 1997). Subsequently, receptors are sorted into distinct early endosomal sub-domains and transported to their intended sub-cellular destination through coordinated vesicle shuttling (whereby the cargo-containing endosome fuses with existing organelles or complex vesicles) and maturation (whereby effector and adaptor molecules join the cargo-containing vesicle to alter its characteristics) (Thilo et al., 1995).

**Recycling**

Receptor recycling back to the cell surface following endocytosis is generally understood to occur via two overlapping pathways distinguished by the time taken for the receptor to reappear at the plasma membrane and the organelles involved in sorting. Receptors recycling via the “rapid” pathway replenish the plasma membrane directly from early endosomes within minutes (eg. β2-adrenergic receptor (β2ARs), Tsao and von Zastrow, 2000b; oxytocin receptor, Conti et al., 2009; vasopressin 1a receptor, Innamorati et al., 1998), whereas “slowly” recycling receptors travel via a “perinuclear recycling compartment” and repopulate the surface in a matter of hours (eg. angiotensin II type 1A receptor, Dale et al., 2004; endothelin-1 A receptor, Bremnes et al., 2000; M4 muscarinic acetylcholine receptor, Volpicelli et al., 2002).
The recycling route taken may be associated with the β-arrestin type that interacts with the receptor during endocytosis and the duration of this interaction. Class A GPCRs interact only transiently with β-arrestin 2 and tend to recycle rapidly, whereas class B receptors remain associated with β-arrestin 1 or 2 during endocytosis and can be colocalised with β-arrestin in the perinuclear recycling compartment (Oakley et al., 2000). Some receptors usually noted to undergo rapid recycling appear to also recycle via the slow route under certain circumstances (eg. Moore et al., 1999a).

As well as being expressed at the surface of cells, a number of receptors exhibit a significant degree of cytoplasmic localisation under basal conditions. For at least some of these receptors, the “intracellular pool” appears to be formed by tonically internalising receptors that are transiently sequestered in the cytoplasm but may be recycled back to the cell surface constitutively or on-demand to aid in resensitisation. While there is direct evidence for this constitutive recycling pathway for some receptors (eg. Shapiro and Coughlin, 1998; Parent et al., 2001; Miserey-Lenkei et al., 2002), others appear to be delivered directly to the intracellular pool from the synthetic pathway. In this case, plasma membrane insertion is stimulated following activation of surface receptors, presumably as an alternative mechanism to recycling that promotes rapid resensitisation (eg. Hein et al., 1994; Sengelov et al., 1994).

Degradation

While all cellular protein is likely to undergo turnover and therefore eventually be subject to proteolytic degradation, a number of receptors exhibit little or no post-endocytic recycling and instead are directed towards degradative pathways following endocytosis (eg. protease activated receptor 1, Trejo et al., 1998; δ opioid receptor, Tsao and von Zastrow, 2000b; endothelin-1 B receptor, Bremnes et al., 2000). This phenotype tends to promote tolerance to the receptor ligand as new receptor synthesis is required to repopulate the plasma membrane (Tappe-Theodor et al., 2007), although as mentioned above, mobilisation of intracellular pool receptors may be a mechanism for more rapid resensitisation of some receptors. Receptor
degradation is typically observed over a number of hours following initial agonist application and eventuates in lysosomal proteolytic degradation. Some latency in this process is perhaps due to the steps taken in maturation of early endosomal vesicles to more acidic late endosomes, which contain cisternal and tubular regions with numerous membrane invaginations (Katzmann et al., 2002).

Interestingly, chronic agonist stimulation leads to marked downregulation of the majority of receptors, including those normally classified as “recycling”. For example, $\beta_2$ARs undergo repeated endocytosis and recycling but are eventually downregulated after prolonged agonist stimulation (Morrison et al., 1996; Moore et al., 1999b). As these studies have been carried out on transfected receptor expressed under a constitutive promoter, this is unlikely to be due to transcriptional regulation. This phenomenon instead seems to be due to a small fraction of receptors being degraded in each cycle, which is perhaps controlled by an as yet unidentified tagging mechanism that allows the cell to monitor the history of individual receptor units (Moore et al., 1999b).

**Synthesis and delivery**

The preceding sub-sections focused on endocytosis and post-endocytic trafficking, however a crucial determinant of the potential for trafficking via these pathways is the delivery of receptors to the appropriate cellular compartment following synthesis.

Following transcription of mRNA, translation proceeds at the endoplasmic reticulum (ER) via ER-attached ribosomes. Correct receptor folding and translocation across the ER membrane is crucial for receptor stability and ultimate function as misfolded receptor is detected by ER “quality control” pathways and proteolytically degraded, usually via the ubiquitin-proteasome system (reviewed in Achour et al., 2008). The receptor N-terminus is often co-translationally translocated across the ER membrane, however this may also occur at the conclusion of translation (Kanner et al., 2002). A number of GPCRs with long N-terminal tails (average 200
amino acids) possess signal peptides that are translated prior to the receptor sequence proper to facilitate ribosome-ER attachment and N-terminal translocation through interaction with signal recognition peptides and their receptors (Wallin and von Heijne, 1995). Recognition sequences in the receptor transmembrane regions associate with distinct components of the ER translocon and assist in folding the remaining receptor peptide into its correct seven transmembrane-spanning conformation (Meacock et al., 2002). Among other modifications, many GPCRs are glycosylated on specific N-terminal tail and extracellular loop residues which may assist with correct receptor folding and/or eventual targeting and function (Wheatley and Hawtin, 1999; Lanctot et al., 2005). Folded and glycosylated receptors are transported via coat protein complex II (COPII)-coated vesicles to the Golgi apparatus and move through the cis- and medial-Golgi compartments where further post-translational modification occurs (reviewed in Achour et al., 2008). Finally, cargo proteins are sorted in the trans-Golgi and delivered to the appropriate plasma membrane domain or organelle (reviewed in Gu et al., 2001).

Control and modulation of receptor trafficking

As may be expected from the cell’s ability to segment different receptor types into diverse intracellular fates, a number of mechanisms for controlling receptor trafficking exist. As well as a range of adaptor and effector proteins that appear to serve in the regulation or execution of trafficking for many receptors, a variety of protein sequence motifs have recently been identified that suggest the existence of an even greater level of complexity than previously realised.

Adaptor and effector molecules

A wide range of proteins that contribute to vesicular transport have been identified. These include coat proteins (eg. clathrin and caveolin as already mentioned) and docking and fusion mediators (eg. Soluble NSF Attachment Protein Receptors; Sollner et al., 1993). While a few of these are associated with particular trafficking pathways, for the most part these perform fairly ubiquitous roles.
A family of trafficking adaptors with more specificity for trafficking pathways is the “Ras-like from brain” (Rab) GTPases. Although only a few of the 60 or so family members have been studied thoroughly (Simpson and Jones, 2005), the well-characterised members have become widely utilised in receptor and other integral membrane protein trafficking studies to assist in the definition and clarification of relevant trafficking pathways. The regulatory capabilities of Rab proteins stem from their ability to switch between GTP- and GDP-bound states. In GDP-bound form, Rab GTPases are typically complexed with Rab GDP-dissociation inhibitors (GDI) in the cytosol. The Rab acquires its active, membrane-bound form when GDI-displacement factors and guanine exchange factors (GEFs) catalyse replacement of GDP with GTP. Once activated, Rabs are recognised by effector molecules that facilitate the formation of adaptor and effector protein complexes and vesicle tethering, motility, fusion and budding. Activity is halted when a GTPase activating protein (GAP) promotes GTP hydrolysis and the Rab protein returns to its inactive form (reviewed in Stenmark, 2009). Rab protein activity is also regulated by phosphorylation (Ayad et al., 1997; Chiariello et al., 1999).

Based on characterisation of Ras nucleotide binding and hydrolysis, mutations of the analogous motifs in Rab proteins have been identified that produce GTP-bound constitutively active, or GDP-bound inactive forms. These have been useful in both elucidating the functions of the Rabs and in clarifying receptor trafficking via the pathways they control. Thereby, Rab family members known to mediate GPCR trafficking include Rab5 (endocytosis and early endosome fusion), Rab4 (early endosomal sorting, particularly to recycling pathways), Rab11 (slow recycling via the perinuclear recycling compartment) and Rab7 (trafficking to late endosomes and lysosomes) (reviewed in Stenmark, 2009).

More recently discovered adaptor proteins that appear more specific to GPCR trafficking and bind directly to receptors include the PDZ domain-containing protein and GPCR-associated sorting protein (GASP) families (reviewed in Hanyaloglu and Zastrow, 2008). These also interact with multiple receptors to control their trafficking, for example GASP1 mediates
lysosomal degradation of at least three GPCRs (Whistler et al., 2002; Bartlett et al., 2005; Martini et al., 2007).

Not as much is known about the molecular mechanisms controlling receptor delivery to the cell surface following synthesis (Duvernay et al., 2005). One interesting “partner” protein, ubiquitin-specific protease Usp4, de-ubiquitinates adenosine 2A receptors in the ER and enhances their delivery to the plasma membrane (Milojevic et al., 2006). Pharmacological chaperoning represents a further developing area of research in which the application of receptor ligands or other molecules with affinity for the receptor can be effective in facilitating correct receptor folding, or perhaps even repairing misfolded protein (reviewed in Bernier et al., 2004; Petäjä-Repo and Bouvier, 2005).

Receptor motifs and recognition sequences

Surface repopulation via the rapid recycling pathway was once assumed to represent a default pathway occurring by bulk membrane flow. That is, receptors would recycle to the cell surface unless a specific cellular signal redirected the receptors to a degradation pathway (Koenig and Edwardson, 1997). However, this idea of “default” recycling was challenged by Cao et al. in 1999 when a PDZ protein interaction domain (DSLL) sorting motif in the carboxy-terminal tail of the β₂AR was found and determined to control endocytic sorting via interaction with other cytosolic proteins. Interaction between this receptor domain, ERM (ezrin-radixin-moesin)-binding phosphoprotein 50 (EBP50) and the actin cytoskeleton were found to be required for efficient β₂AR recycling. Subsequent studies have confirmed that this PDZ domain can function as an autonomous sorting signal through the demonstration that appending the sequence onto a normally degradative receptor could reroute it to a recycling pathway (Gage et al., 2001).

Since the discovery of this signal sequence in the β₂AR, similar trafficking sequences have been discovered in the human lutropin receptor (Galet et al., 2003), endothelin-1 A receptor
(Paasche et al., 2005), μ opioid receptor (Tanowitz and von Zastrow, 2003) and dopamine receptor 1 (D1; Vargas and Von Zastrow, 2004). To date, some trafficking signals are conserved, for example PDZ motifs are present in a number of GPCRs (Paasche et al., 2005), however others are not conserved between receptor types, nor are they always localised in the distal cytoplasmic tail (Vargas and Von Zastrow, 2004). Such observations are indicative that receptor trafficking is under tight control and may be specific for particular receptor types.

Another study has investigated commonalities and divergence in trafficking regulation by screening a library of GPCR carboxy-terminal tails for interactions with proteins known to be involved in directing certain receptors to recycling or degradative states (Heydorn et al., 2004). Of the 59 represented receptors, EBP50 (described above) bound only to the β2AR tail. Three other proteins involved in trafficking to recycling or degradative fates were also investigated, each of which bound to at least ten of the tails contained in the library studied. Thus it seems that these proteins may contribute to the control of trafficking for a range of GPCRs, whereas EBP50 may represent an adaptor specific to the DSLL sequence in β2AR. GASP1 also bound to the β2AR, reinforcing the seemingly “dual-fate” nature of this receptor, and that the identity of the adaptor protein interacting with a receptor at any one time determines the trafficking pathway travelled (Heydorn et al., 2004).

Receptor oligomerisation

While classical paradigms of GPCR function consider receptors to be individually functioning units, an increasing body of evidence suggests that many, if not most, GPCRs physically interact with one or more units of the same or a differing type to form homo- or hetero-oligomers respectively. These oligomers can exhibit altered ligand binding and/or signalling properties and hold potential as novel therapeutic targets towards which bivalent ligands may be designed to facilitate selective targeting of a sub-population of the individual receptor constituents (reviewed in Zeng and Wess, 2000; Bouvier, 2001; Milligan, 2001). For some GPCRs, oligomerisation is critical for surface expression of functionally active receptors (eg.
Kuner et al., 1999; Lee et al., 2000). Although only a small number of studies related to GPCR oligomerisation and post-endocytic trafficking have been performed thus far, both homo- and hetero-oligomerisation have been shown to influence trafficking (Terrillon et al., 2004; Cao et al., 2005).

**GPCR trafficking in disease**

Disruptions in GPCR trafficking have been implicated in a variety of diseases. The majority of described disruptions are due to mutations in the receptor sequence which lead to aberrant retention in the cytoplasm. These include retinitis pigmentosa caused by ER trapping of misfolded mutant rhodopsin (Sung et al., 1991) and nephrogenic diabetes insipidus caused by mutation and disrupted surface delivery of the vasopressin type 2 receptor (Birnbaumer et al., 1994). Interestingly, such mutations often also alter the trafficking of wild-type receptor and thereby act in an autosomal dominant manner (reviewed in Ulloa-Aguirre et al., 2004). This likely occurs through oligomerisation of wild-type with mutant receptor and suggests that potential hetero-oligomeric partners might also be affected in a heterologous manner (reviewed in Conn et al., 2007). It is important to note that a number of GPCR mutations are pathogenic only for reasons of receptor mis-location and in fact intrinsic receptor function is retained. The use of pharmacological chaperones to correct mis-routing and rescue function is therefore an exciting prospect and has shown promise in initial clinical trials (eg. Bernier et al., 2006).

Mutation or abnormal expression of some trafficking adaptor proteins have also been identified as causative in disease. These include a few members of the Rab GTPase family which have been implicated in Griscelli syndrome and some types of cancer (Ménasché et al., 2000; Cheng et al., 2005). Aberration of intracellular trafficking is also likely to play a central role in Huntington’s disease which is caused by a mutation in huntingtin, a protein associated with a variety of trafficking pathways (reviewed in Hanyaloglu and Zastrow, 2008). As our understanding of the molecular mechanisms controlling receptor trafficking advances it is likely that dysregulation or mutation of trafficking adaptor proteins will increasingly be identified as
pathogenic mechanisms, reinforcing the importance of continued research into receptor trafficking.

CANNABINOID RECEPTOR 1 FUNCTION, PHARMACOLOGY AND INTRACELLULAR TRAFFICKING

The endocannabinoid system consists of endogenously synthesised cannabinoid compounds, the enzymes that produce and metabolise them, and the receptors that transduce their effects.

Two principal endocannabinoids have been described: anandamide (Devane et al., 1992) and 2-arachidonyl glycerol (Mechoulam et al., 1995). These are synthesised by the cleavage of plasma membrane phospholipids, on demand at or near the site of action (Di Marzo et al., 1994), however it has not yet been definitively elucidated as to whether endocannabinoid delivery to, or removal from the site of action, is facilitated by active transfer or passive diffusion. A number of enzymes that are responsible for endocannabinoid synthesis and metabolism have been identified. Various other putative endocannabinoid compounds have also been proposed although they are yet to be extensively characterised (reviewed in Di Marzo, 2008).

Two class A (Rhodopsin-like) GPCRs have been identified as the predominant mediators of endo- and exogenous cannabinoid effects. CB₁ (Matsuda et al., 1990; Gerard et al., 1991) is generally referred to as the brain-type cannabinoid receptor due to its particularly high levels of expression in the central nervous system, whereas Cannabinoid Receptor 2 (CB₂; Munro et al., 1993) is principally expressed in blood-borne immune cells and related tissues (Galiègue et al., 1995). However, CB₁ is also found at various sites in the periphery, and CB₂ has recently been identified in the brain; most likely being expressed in glial immune cells (reviewed in Pazos et
al., 2004) but perhaps also on neurons (eg. Van Sickle et al., 2005). Other receptors, including GPR55 and transient receptor potential vanilloid types 1, 2 and 4, may also play roles in mediating cannabinoid function although debate continues as to their appropriate classification within the endocannabinoid system (reviewed in De Petrocellis and Di Marzo, 2009). Cannabinoids may also exert non-receptor-mediated effects, such as anti-oxidant activity, particularly when applied at high concentration (eg. Chen and Buck, 2000).

As is clear from the effects of cannabis, and is now being exploited therapeutically, the endocannabinoid system can be influenced by exogenous compounds. A plethora of chemicals with the potential for activity in the cannabinoid system exist in nature, for example more than 60 have been identified in *Cannabis sativa*, including Δ9-tetrahydrocannabinol, a major psychoactive and immunomodulatory component of marijuana (reviewed in Ashton, 2001). The development of synthetic cannabinoids with greatly enhanced potency and stability compared with cannabis-derived compounds and endocannabinoids, some of which have been converted to radioligands, have greatly enhanced the potential and scope for research into the cannabinoid system and are valuable research tools. All known naturally occurring cannabinoid compounds are extremely hydrophobic, and therefore cross the blood-brain barrier easily (McGilveray, 2005), however hydrophilic non-blood-brain barrier permeant ligands have recently been developed and may prove useful in modulating peripheral cannabinoid receptor function while avoiding central side-effects (Thakur et al., 2009).

As the research described in this thesis is concerned with CB1 intracellular trafficking, the remainder of this introduction will focus on CB1 function.

*In vivo functions and implications in disease*

CB1 is one of the most abundant GPCRs in the mammalian central nervous system (Herkenham et al., 1991; Herkenham et al., 1990). Regional localisation is well characterised, with particularly high CB1 levels noted in the basal ganglia, hippocampus, cortex, amygdala and
spinal cord (eg. Herkenham et al., 1990; Herkenham et al., 1991; Glass et al., 1997). These regions of expression in the brain correlate with the functions modulated by receptor activation: movement and coordination, memory, executive functioning, mood and nociception respectively (reviewed in Breivogel and Sim-Selley, 2009). The overall absence of CB1 from the brain stem is thought to explain the low risk of lethality from cannabis consumption (Herkenham et al., 1990; Glass et al., 1997).

In neurons, CB1 is expressed at the pre-synaptic plasma membrane, while endocannabinoids are released from the post-synaptic side in response to neuronal depolarisation (Katona et al., 1999). CB1 activation usually inhibits the release of co-expressed neurotransmitters and thereby suppresses their activity. This phenomenon is known as depolarisation-induced suppression of excitation or inhibition, depending on the classification of the affected neurotransmitter (Wilson and Nicoll, 2001). CB1-mediated signalling cascades also induce changes in gene expression which are associated with direct effects on cell physiology and include apoptosis, differentiation and proliferation. However, the exact effects seem highly dependent on regional and temporal context. For example, CB1 activation has been shown to both protect against and stimulate apoptosis (reviewed in Velasco et al., 2005).

In the periphery, CB1 is expressed at various sites including the gastrointestinal tract, pancreas, liver, kidney, prostate, testis, uterus, eye, lungs, adipose tissue and heart (Galiègue et al., 1995). CB1 activity in these regions is associated with energy balance, metabolism, nociception and cardiovascular health (reviewed in Mackie, 2008). As well as being present at the plasma membrane, significant cytoplasmic expression is observed in both neuronal and non-neuronal cells (eg. Pettit et al., 1998; Tsou et al., 1998; Katona et al., 1999; Hsieh et al., 1999; Leterrier et al., 2004).

Abnormalities in CB1 function have been implicated in a number of neurological disorders including Huntington’s disease (early and preferential loss of CB1), Alzheimer’s disease (loss
and/or atypical localisation of CB₁), schizophrenia (CB₁ gene polymorphisms associated with particular symptom pedigrees) and depression (decreased CB₁ expression and links with CB₁ gene polymorphisms) (reviewed in Maccarrone et al., 2007; Onaivi, 2009). The mechanisms via which cannabinoid system dysfunctions influence pathogenesis have not been fully elucidated, and it is not always clear as to whether the abnormal state of the cannabinoid system is causative, symptomatic or compensatory in these diseases. Initial results from administration of CB₁ agonists or inverse-agonists in disease models and clinical trials have been mixed, nonetheless research continues and alternative approaches, such as modulation of endocannabinoid metabolising enzymes, may be useful in increasing the specificity of drug effects (Makriyannis et al., 2005; Kunos et al., 2009). However, a number of CB₁-targeted drugs are approved or show promise as supportive and symptomatic therapies for conditions such as amyotrophic lateral sclerosis (activation may reduce spasticity and excitotoxicity), Parkinson’s disease (activation or blockade may be neuroprotective depending on the cell type and model), Tourette’s syndrome (activation may suppress motor and behavioural symptoms), epilepsy (activation is generally found to be anti-convulsant), glaucoma (activation reduces intraocular pressure), cancer (activation reduces cell proliferation), multiple sclerosis (activation is analgesic and may reduce motor symptoms and excitotoxicity) and drug addiction (blockade may interfere with drug reward pathways) (reviewed in Makriyannis et al., 2005; Maccarrone et al., 2007; Kogan and Mechoulam, 2007). CB₁ also plays an important role in weight management both centrally and peripherally. The cannabinoid agonist Dronabinol is currently approved to treat nausea and stimulate appetite in chronic disease and patients undergoing cancer chemotherapy, and may also be useful in treating anorexia nervosa (Kogan and Mechoulam, 2007). Although the CB₁ inverse-agonist Rimonabant (SR 141716A) was recently withdrawn from the market as an anti-obesity therapy due to adverse psychological effects (anxiety and suicidal thoughts), newly developed non-blood-brain barrier permeable CB₁ inverse-agonists are likely to confer beneficial peripheral effects (including increased energy expenditure in adipocytes and inhibition of lipogenesis in the liver) without central side-effects (Kunos et al., 2009).
Signalling

Much cannabinoid research has focused on determining the ways in which CB₁ transduces intracellular signals and thus effects changes in cell function and phenotype. CB₁ primarily couples to Gαᵢ, thus stabilisation of the receptor in its active state inhibits adenylate cyclase activity and the accumulation of cyclic adenosine monophosphate (Howlett, 1984). Downstream effects of this interaction include the activation of inwardly rectifying potassium channels (Mackie et al., 1995) and the regulation of cAMP-dependent enzymes (Davis et al., 2003). Other consequences of CB₁ activation, likely mediated by the G-protein βγ subunits, include the inhibition of calcium channels (Caulfield and Brown, 1992), and the induction of immediate early gene expression, such as Krox 24 (Graham et al., 2006). As for the majority of GPCRs, prolonged presence of cannabinoid agonists leads to desensitisation. This event is mediated by GRK-3 phosphorylation which facilitates β-arrestin 2 binding and steric hindrance of G-protein complex interactions, thus preventing initiation of intracellular signal cascades (Jin et al., 1999).

CB₁ has been referred to as a “promiscuous” receptor in that affinity for and/or activity via Gₒ, Gₛ, and Gₐα subtypes have also been demonstrated. Such interactions may be physiologically mediated via differential expression or compartmentalisation of G-protein complexes, hetero-oligomerisation with other GPCRs, or ligand-selective G-protein association and it is likely that the diversity in the behavioural effects induced by cannabinoids are partly mediated by the activation of several distinct intracellular signalling pathways (Breivogel et al., 1997; Glass and Felder, 1997; Glass and Northup, 1999; Kearn et al., 2005; Lauckner et al., 2005). In addition to traditional signalling cascades, additional mechanisms for complexity in CB₁-mediated cellular responses continue to be discovered. A recent finding was that the affinity of ligands at the CB₁ orthosteric site, and/or efficacy of the transduced response, may be modulated via interaction of molecules at an allosteric site (Price et al., 2005; Navarro et al., 2009). This will certainly be an interesting area to follow as further evidence is gained as to the signalling modulation that can be achieved and potential therapeutic applications (Ross, 2007).
A significant degree of constitutive CB₁ activity has also been observed (e.g. Bouaboula et al., 1997). That is, the receptor exhibits a degree of tonic activation, and thus downstream signalling, in the absence of agonist. The majority of cannabinoid compounds originally considered antagonists have since been found to inhibit this constitutive signalling and are therefore now termed inverse-agonists (e.g. Landsman et al., 1997). However, as highlighted by a recent study, it is important to keep in mind that cells or model systems expressing cannabinoid-synthesising enzymes may produce endocannabinoids and falsely give the impression of constitutive activation and inverse-agonism through competition with the cannabinoids present in the experimental assay (Turu et al., 2007). Studies to directly investigate the prevalence of this type of phenomenon are complicated by the structural similarities of enzyme inhibitors with receptor ligands; thereby the inhibitors may also have affinity for cannabinoid receptors. Although a few neutral antagonists (ligands that competitively bind at CB₁ but do not alter constitutive signalling) have been generated, these have yet to be rigorously investigated (Pertwee, 2005).

An additional point of speculation, given that cannabinoid ligands are lipophilic and can cross cell membranes, has been whether CB₁ residing in the cytoplasm could contribute to functional responses. One study, utilising a recently developed non-lipophilic inverse-agonist in a neuronal cell line expressing CB₁ endogenously, indicated that this is indeed the case. However, the findings were somewhat controversial in that surface receptors apparently did not contribute to the signalling response (Rozenfeld and Devi, 2008). These findings have yet to be replicated in a different model system.

**Intracellular trafficking**

**Structure and nascent processing**

Although reductions in CB₁ mRNA levels have been identified as a potential pathogenic mechanism for CB₁ downregulation (reviewed in Maccarrone et al., 2007), only a few studies
have directly addressed aspects of transcriptional regulation and mRNA processing. While transcription start sites and non-coding sequences upstream of the coding region have been identified, the promoter sequence has not been definitively described (McCaw et al., 2004; Zhang et al., 2004). As well as the most commonly referred to and widely studied 472-residue CB₁ isoform (sometimes referred to as the “long” isoform), two alternative mRNA splice variants have been identified. CB₁a is 411 amino acids long and contains a 28-residue span in the N-terminus that differs from the full-length isoform (Shire et al., 1995), and CB₁b is truncated at the N-terminus by 33 amino acids (Ryberg et al., 2005). As these splice variant mRNA species are present at much lower levels than full-length CB₁ in various tissues and the receptors they encode have low affinity for cannabinoid agonists (Rinaldi-Carmona et al., 1996; Ryberg et al., 2005), the *in vivo* role and significance of these isoforms is unclear.

Once transcribed to mRNA the nascent CB₁ polypeptide is synthesised and translocated across the ER membrane to facilitate receptor folding and assume a typical GPCR seven transmembrane-spanning conformation. GPCRs with long N-terminal tails often have a cleavable signal sequence which facilitates membrane translocation, however CB₁ does not, despite possessing a long N-terminal tail (116 amino acids; Wallin and von Heijne, 1995). The addition of a signal sequence to a heterologously expressed CB₁ construct markedly increased overall receptor expression levels, likely because more nascent receptor species were successfully translocated and therefore escaped ER quality control mechanisms that would normally result in the degradation of non-translocated receptor (Andersson et al., 2003). Mutation of the N-terminal tail to shorten it to 27 amino acids in length improved receptor stability and surface expression similarly to the signal sequence construct, and interestingly did not have any apparent effect on ligand binding (Andersson et al., 2003). These observations correlate with findings for endogenously expressed CB₁ in N18TG2 neuroblastoma cells, whereby the majority of newly synthesised CB₁ was degraded rapidly with a measured half-life of just under 5 hours, whereas the apparently functional pool of receptors had a half-life of more than 24 hours (McIntosh et al., 1998).
Once the N-terminal tail is successfully translocated across the ER membrane it is N-glycosylated at two distinct sites (Song and Howlett, 1995). A putative palmitoylation site in the C-terminus has been suggested to anchor the cytoplasmic tail to form a fourth intracellular loop (Mukhopadhyay et al., 1999), however this modification has not yet been demonstrated experimentally. Little is known about the specific mechanisms regulating ER and Golgi export of CB₁, however it appears that conserved structure of the helix 8 region in the C-terminal tail is essential for efficient export from the ER (Ahn et al., 2010).

**Agonist-induced and constitutive internalisation**

As for the majority of GPCRs, CB₁ undergoes internalisation following agonist binding. Endocytosis is rapid in transfected cell lines (e.g. Rinaldi-Carmona et al., 1998; Hsieh et al., 1999; Daigle et al., 2008b), however proceeds at a slower rate in primary cultured neurons (Coutts et al., 2001; Leterrier et al., 2006). The mechanisms giving rise to this difference have not been identified, however may be indicative of differential trafficking according to cell type or plasma membrane sub-domain (Koenig and Edwardson, 1997; Keren and Sarne, 2003). Endocytosis is generally understood to occur via classical clathrin-coated pits (Hsieh et al., 1999; Daigle et al., 2008b; Wu et al., 2008), and is mediated by Rab5 (Leterrier et al., 2004) and phospholipase D2 (Koch et al., 2006). However in at least some cell types, CB₁ internalises via caveolae, and association with lipid rafts may also influence ligand binding and/or signalling (Keren and Sarne, 2003; Bari et al., 2008; Wu et al., 2008).

Also as for other receptors, CB₁ signalling and endocytosis are closely related, but not dependent on one another. This has been demonstrated through multiple approaches; internalisation is not influenced by blockade of signalling via Gαᵢ and Gαₛ with pertussis or cholera toxins (Hsieh et al., 1999; Coutts et al., 2001), and blockade of internalisation with generalised endocytosis inhibitors or mutation of particular amino acid residues does not influence signalling via classical G-protein cascades (Roche et al., 1999; Daigle et al., 2008b). β-arrestin 2 is involved in CB₁ desensitisation, however does not appear to be required for
internalisation (Jin et al., 1999). The potential for interaction with β-arrestin 1 does not appear to have been investigated as yet (van der Lee et al., 2009).

Interestingly a CB₁ mutant lacking the terminal 14 amino acids of the cytoplasmic tail exhibited markedly reduced internalisation in AtT-20, but not HEK cells (Hsieh et al., 1999; Daigle et al., 2008b), which is perhaps indicative that a different complement of trafficking adaptor proteins is expressed in the two cell lines. This is exemplified by CRIP1a, a recently identified protein that interacts with the distal CB₁ C-terminus and appears to influence constitutive signalling (Niehaus et al., 2007), which is expressed in AtT-20 but not HEK cells. It was therefore supposed that CRIP1a may regulate CB₁ internalisation (Daigle et al., 2008b), however this has yet to be tested experimentally.

Constitutive internalisation has also been observed and is also associated with, although not dependent upon, constitutive activation (Leterrier et al., 2004; Leterrier et al., 2006; McDonald et al., 2007). Concordantly, application of inverse-agonist to prevent constitutive signalling and internalisation results in surface receptor upregulation (Rinaldi-Carmona et al., 1998; Leterrier et al., 2004). Tonic endocytosis has been postulated to be responsible for accumulation of CB₁ in the cytoplasm (Leterrier et al., 2004). This concept is expanded upon below.

Recycling

As mentioned above, in the brain, CB₁ is expressed at the pre-synaptic cell membrane of neuronal axon terminals (eg. Katona et al., 1999; Kawamura et al., 2006). Plasma membrane expression in primary neuronal cultures is associated with axonal processes (Coutts et al., 2001; Leterrier et al., 2006; McDonald et al., 2007), and is also present in endogenously expressing (McIntosh et al., 1998; Graham et al., 2006) and transfected (Rinaldi-Carmona et al., 1998; Hsieh et al., 1999; Leterrier et al., 2004; Tappe-Theodor et al., 2007; Wu et al., 2008) immortalised cell lines. Interestingly, detailed ultrastructural studies have revealed that in vivo a significant proportion of CB₁ is located in a cytoplasmic “intracellular pool” (eg. Pettit et al.,
1998; Tsou et al., 1998; Katona et al., 1999). *In vitro* models also exhibit this distribution (eg. McIntosh et al., 1998; Rinaldi-Carmona et al., 1998; Hsieh et al., 1999; Leterrier et al., 2004; Graham et al., 2006), one study estimating that 85% of total cellular CB1 was located intracellularly (Leterrier et al., 2004). The source and function of this intracellular pool has been the subject of much speculation.

The observation that CB1 constitutively endocytoses (Leterrier et al., 2004) and correlation of results with other receptors that exhibit similar phenotypes (eg. Parent et al., 2001; Miserey-Lenkei et al., 2002; Marion et al., 2004) have led to the inference that this intracellular pool serves as a reservoir of endocytic origin. This reservoir may function as a source from which surface CB1 is replenished to replace internalised receptor (Leterrier et al., 2004), inferring that CB1 exhibits a recycling phenotype. In line with this hypothesis, application of inverse-agonist appears to re-distribute intracellular CB1 to the cell surface and this process was shown to be mediated by Rab4, a marker of rapid recycling pathways (Leterrier et al., 2004). In primary cultured neurons transiently expressing GFP-tagged CB1, this constitutive cycle appears to be responsible for domain-specific CB1 expression. Internalisation blockade experiments indicated that receptors are delivered to the somatic membrane, however subsequently undergo constitutive endocytosis and are delivered to the axonal membrane (Leterrier et al., 2006; McDonald et al., 2007). Consistent with these theories, intracellular pool CB1 displays only minimal colocalisation with protein synthesis-associated organelles in both transfected cells and those that endogenously express CB1 (Leterrier et al., 2004; Rozenfeld and Devi, 2008).

CB1 may also recycle following agonist-induced internalisation, as has been demonstrated by immunocytochemistry in cell lines either in the presence of a protein synthesis inhibitor (to exclude the possibility that surface re-population is a result of new receptor synthesis) (Hsieh et al., 1999; Tappe-Theodor et al., 2007) or following selective labelling of surface receptors in order to follow their endocytic fate (Martini et al., 2007). Detection of recycling appeared to be
dependent on replacement of agonist with inverse-agonist and was blocked by an inhibitor of vesicle acidification (Hsieh et al., 1999). Long (90 minutes as opposed to 20) or high-concentration agonist stimulations appeared to divert CB1 to a degradative rather than recycling pathway, leading to the hypothesis that CB1 is a “dual-fate” receptor; that is, exhibiting a recycling or degrading phenotype depending on the exact stimulation conditions or cellular context in a similar manner to the β2AR (Hsieh et al., 1999; Martini et al., 2007). However, recycling appears to be significantly slower for CB1 in comparison with the β2AR, requiring in the order of hours to repopulate the cell surface (Hsieh et al., 1999; Martini et al., 2007). This time scale is more consistent with the Rab11-associated “slow” recycling pathway, than the Rab4 “rapid” route, yet Rab4 but not Rab11 has been shown to influence CB1 trafficking (Leterrier et al., 2004).

Degradation

Further to the observations in experiments designed to measure receptor recycling, downregulation of CB1 following chronic agonist stimulation has also been widely reported both in vivo (Oviedo et al., 1993; Breivogel et al., 1999) and in vitro (Hsieh et al., 1999; Martini et al., 2007; Tappe-Theodor et al., 2007). This phenomenon has been linked with the development of tolerance to cannabinoid ligands (Tappe-Theodor et al., 2007). Interestingly, it is rarely reported that the entire cellular population of CB1 can be induced to degrade with chronic agonist treatment, an observation which may lend support to the “dual-fate” classification if the remaining non-degraded receptors retain the potential for recycling. Inhibitor and colocalisation studies suggest post-endocytic CB1 degradation occurs primarily via lysosomal proteolysis (Martini et al., 2007).

Recently GASP1 (Martini et al., 2007; Tappe-Theodor et al., 2007) and AP3 (Rozenfeld and Devi, 2008), adaptor proteins associated with sorting and delivery of receptors to lysosomes, were demonstrated to colocalise and directly interact with CB1. Furthermore, interruption of the GASP1 interaction with a dominant-negative mutant prevented degradation in cell and mouse
models (Martini et al., 2007; Tappe-Theodor et al., 2007), and a knockout GASP1 mouse model exhibited reduced behavioural tolerance to chronic agonist administration (Martini et al., 2010), providing additional evidence towards the theory that CB₁ degradation contributes to the development of tolerance to cannabinoid ligands.

Seemingly in opposition to the theory that CB₁ recycles, recent reports demonstrate striking colocalisation of internalised CB₁ and the intracellular pool with degradative pathway markers (lysosomal-associated protein 1 (LAMP1), Lysotracker® (Invitrogen) and Rabs7 and 9) but lack of colocalisation with rapid recycling pathway marker transferrin (Martini et al., 2007; Rozenfeld and Devi, 2008). These results suggest that, at least under certain conditions, CB₁ may be preferentially degraded and the intracellular pool might in fact represent a reservoir of receptors destined for degradation, rather than recycling. Observations of prolonged tolerance to behavioural effects and signalling responses following cannabinoid drug administration suggest that new receptor synthesis may be required to restore cannabinoid sensitivity and lend support to this theory. Regional differences in recovery of responsiveness may be indicative of differential regulation between cell populations (Bass and Martin, 2000; Sim-Selley et al., 2006; reviewed in Gonzalez et al., 2005).

**Influence of oligomerisation**

The potential for CB₁ dimerisation with other GPCRs has been demonstrated, as well as the propensity of such interactions to influence signal transduction (eg. Glass and Felder, 1997). Only a few studies have investigated the effects of oligomerisation on CB₁ trafficking, however it appears that orexin-1 and dopamine D₂ receptors may both hetero-oligomerise with CB₁ and influence each other’s trafficking phenotype (Ellis et al., 2006; Przybyla and Watts, 2010). Such observations suggest that the relative levels of co-expressed receptors in different model systems and cell types may assist in explaining some of the variability in CB₁ trafficking phenotypes noted to date and further investigation is certainly warranted.
AIMS AND HYPOTHESES

The aims and hypotheses addressed in this thesis have evolved to some extent over the course of the research. Based on evidence in the existing literature, the project was founded on the hypothesis that CB₁ undergoes recycling following constitutive or agonist-induced internalisation. It was intended that this pathway be demonstrated and, facilitated by a combination of receptor mutagenesis and two-hybrid screening techniques, potential adaptor proteins important for CB₁ recycling investigated.

However, the results of initial experiments were perplexing as CB₁ internalisation and degradation were observed, but recycling was not. As repeated attempts to confirm the recycling hypothesis were unsuccessful, increasing evidence was gathered to support the null hypothesis: that CB₁ does not recycle. These findings also illuminated the predominantly indirect nature of the existing evidence, and ultimately call for a revision of the general understanding of CB₁ trafficking indicated by the current literature.

Having come to these realisations, the research presented in this thesis addresses the following objectives:

Aim One: Develop a quantitative method for studying receptor trafficking.

Although a variety of techniques for studying receptor trafficking exist in the literature, the majority were not suitable for carrying out aims two and three (below). The availability of the Discovery-1™ automated imaging and analysis platform at the facility where this research was carried out provided the opportunity to develop an immunocytochemistry-based high-throughput and high-content quantitative assay with the potential to address many of the limitations of previously published methods. Ideally, the new method would facilitate accurate and sensitive quantification of receptors in various cell compartments at high-throughput and with little opportunity for introduced human bias. Other materials and techniques to be
optimised at the outset of the project would be the immunocytochemistry conditions, appropriate concentrations of selected drugs, and a method for selectively detecting intracellular receptors.

**Aim Two: Perform a thorough characterisation of CB₁ trafficking, with particular emphasis on recycling, degradation and the role of the intracellular pool.**

As described above, initial experiments revealed the necessity for a thorough and integrated CB₁ trafficking study. An important precursor to studying post-endocytic trafficking would be to perform a detailed characterisation of agonist-induced and constitutive internalisation. Subsequently, CB₁ recycling and degradation would be studied. It was of particular interest to further investigate the role of the widely reported intracellular pool, which had previously been suggested to form part of a constitutively recycling loop. The majority of studies in the existing literature investigated a single cell model and relied on heterologous expression of CB₁. While for technical reasons this study would also utilise heterologous introduction of CB₁, it was considered a priority to compare these findings in multiple cell types and study endogenously expressed CB₁ in at least one cell line. Additional intended features of this study that would represent improvements on aspects of previous work were the use of antibody “live feeding” techniques to directly investigate *bona fide* recycling, and the independent quantitative measurement of surface versus intracellular CB₁, as opposed to reliance on qualitative microscopy or ratiometric analysis.

**Aim Three: Investigate the role of selected Rab GTPases in CB₁ trafficking.**

Of the evidence for CB₁ recycling in the published literature, the findings of Leterrier et al. (2004) with regard to the influence of selected Rab GTPases on CB₁ basal and ligand-influenced localisation were some of the most difficult to reconcile with the revised theories of CB₁ trafficking proposed as a result of the research in aim two of this thesis. The effects of over-expressing a selection of wild-type, dominant-positive and dominant-negative Rab
GTPases established in the literature to be generally important for receptor internalisation, recycling and degradation were therefore investigated.

Aims one, two and three described above are addressed in chapters three, four and five of this thesis respectively.
Molecular biology

DNA techniques

To propagate DNA for transfection or cloning, DNA was transformed into XL10-Gold ultracompetent bacterial cells (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Transformed bacteria were spread onto 20 g/L LB Broth / 20 g/L Agar (both GE Healthcare, Buckinghamshire, UK) with appropriate selection antibiotics (50 μg/mL ampicillin; 30 μg/mL kanamycin, both Sigma-Aldrich, St Louis, MO). Isolated single colonies were picked from transformation plates and incubated in LB Broth with appropriate selection antibiotics for 16 h at 37°C with shaking to promote aeration. DNA was isolated and purified with Mini-Prep (Qiagen, Hilden, Germany), or Purelink HiPure Midi-Prep (Invitrogen, Carlsbad, CA) kits according to the manufacturer’s instructions. Bacterial cultures were 8 mL for Mini-Prep or 50 mL inoculated with 50 μL from an 8 mL starter culture for Midi-Prep. As required, DNA was visualised by agarose gel electrophoresis.

DNA concentrations were quantified with a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Waltham, MA). For experiments in which DNA concentration was imperative three measurements were taken and averaged. DNA constructs were sequence-verified (DNA Sequencing and Genotyping Facility, School of Biological Sciences, University of Auckland, NZ) using “universal” primers that aligned with sequences in the flanking regions of the plasmid multiple cloning site. Sequencher™ (v. 4.9, Gene Codes, Ann Arbor, MI) and ChromasPro (v.
1.32, Technelysium, Australia) software was used to assess sequencing results and plan cloning strategies.

Receptor constructs

Four receptor constructs were utilised, as listed in Table 2.1.

<table>
<thead>
<tr>
<th>Annotation in text</th>
<th>Receptor species</th>
<th>Tags and/or modifications</th>
<th>Plasmid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-rCB₁</td>
<td>Rattus norvegicus</td>
<td>One haemagglutinin (HA) epitope at receptor N-terminus</td>
<td>pEF4a (Invitrogen)</td>
<td>Prof. Ken Mackie, University of Washington, Seattle, WA; received already transfected into cell line.</td>
</tr>
<tr>
<td>HA-hCB₁</td>
<td>Homo sapiens</td>
<td>Three HA epitopes at receptor N-terminus</td>
<td>pEF4a</td>
<td>#CNR01LTN00, Missouri S&amp;T cDNA Resource Center, <a href="http://www.cdna.org">www.cdna.org</a>. Cloning as noted below.</td>
</tr>
<tr>
<td>pplss HA-hCB₁</td>
<td>Homo sapiens</td>
<td>Preprolactin signal sequence (pplss) and three HA epitopes at receptor N-terminus</td>
<td>pEF4a</td>
<td>CB₁ as above, pplss from Prof. Ken Mackie. Cloning as noted below.</td>
</tr>
<tr>
<td>HA-hD₁</td>
<td>Homo sapiens</td>
<td>Three HA epitopes at receptor N-terminus</td>
<td>pEF4a</td>
<td>#DRD010TN00, Missouri S&amp;T cDNA Resource Center. Cloning as noted below.</td>
</tr>
</tbody>
</table>

Table 2.1 Receptor constructs utilised
The HA-hCB₁ and HA-hD₁ plasmids were purchased in vector pcDNA3.1(+) (Invitrogen). Donor and acceptor (pEF4/V5-His A, Invitrogen) plasmids were digested with KpnI/Pmel (CB₁) or KpnI/XbaI (D₁) restriction enzymes (KpnI and XbaI: Roche, Mannheim, Germany; Pmel: New England Biolabs, Ipswitch, MA), agarose gel purified (QIAquick gel extraction kit, Qiagen) and ligated with T4 DNA ligase (Invitrogen) according to the manufacturers instructions. Use of the KpnI/Pmel restriction enzyme pair resulted in excision of the V5 and poly-His epitopes from the pEF4a vector backbone.

The pplss HA-hCB₁ construct was generated by a colleague (Dr Emma Scotter) for use in a parallel project. Briefly, primers designed with Primer3 (v. 0.4.0, http://frodo.wi.mit.edu/, Rozen and Skaletsky, 2000) were utilised to PCR amplify a 56 base pair pplss coding region from a plasmid gifted to the lab by Prof. Ken Mackie. The PCR reaction included KpnI restriction sites in the flanking regions of the pplss sequence which were subsequently used to insert the pplss into the HA-hCB₁ in pEF4a plasmid, N-terminal and in-frame with the HA epitope sequence.

EGFP-Rab GTPase constructs were also utilised. These are described in detail in chapter five (pg. 118).

Cell culture

Cell lines

The cell lines utilised and DNA constructs they were transfected with (as applicable) are listed in Table 2.2.
### Materials and Methods

#### Table 2.2  Cell lines utilised

<table>
<thead>
<tr>
<th>Annotation in text</th>
<th>Full name</th>
<th>Species and cell type</th>
<th>Transfected plasmid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK</td>
<td>Human embryonic</td>
<td>Homo sapiens, embryonic kidney epithelium with</td>
<td>HA-rCB₁</td>
<td>Prof. Ken Mackie (HA-rCB₁ only), ATCC² #CRL-1573</td>
</tr>
<tr>
<td></td>
<td>embryonic kidney-293</td>
<td>neuronal properties¹</td>
<td>HA-hCB₁, pplss HA-hCB₁</td>
<td></td>
</tr>
<tr>
<td>AtT-20</td>
<td>AtT-20</td>
<td>Mus musculus, pituitary tumour with neuronal properties³</td>
<td>HA-hCB₁</td>
<td>Prof. Ken Mackie (ATCC #CCL-89)</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
<td>Cricetulus griseus, ovary epithelium</td>
<td>HA-hCB₁</td>
<td>ATCC #CRL-9618</td>
</tr>
<tr>
<td>Neuro-2a</td>
<td>Neuro-2a</td>
<td>Mus musculus, neuroblastoma</td>
<td>Not transfected; CB₁ expressed endogenously⁴</td>
<td>ATCC #CCL-131</td>
</tr>
</tbody>
</table>

¹(Shaw et al., 2002); ²ATCC, American Type Culture Collection; ³(Tooze et al., 1989); ⁴(Jordan et al., 2005; Graham et al., 2006).

**Routine maintenance**

Cells were maintained at 37°C / 5% CO₂ in Dulbecco’s modified eagle’s medium (DMEM; Invitrogen) with 10% FBS (NZ-origin, Invitrogen), except the CHO cells which were maintained in DMEM-F12 (Invitrogen) with 10% FBS. Transfected lines were cultured with 250 µg/mL Zeocin™ (Invitrogen) to promote continued heterologous receptor expression. The Neuro-2a cell media was buffered with 25 mM HEPES pH 7.4.
Cells were cultured in 25cm² or 75cm² filter capped, canted neck flasks (BD Biosciences, San Jose, CA). Upon reaching 80-100% confluency (approximately every 2-3 days), cells were sub-cultured with a split of not more than 1:15 for CHO cells or 1:6 for the other cell types. For sub-culturing, cells were rinsed with phosphate buffered saline (PBS; NaCl 1.4 M, KCl 27 mM, Na₂HPO₄ 81 mM, KH₂PO₄ 15 mM), incubated at 37°C with 0.05% trypsin-EDTA (Invitrogen) for 3-5 min, triturated in media and transferred into a new flask with fresh supplemented media.

Cryogenically frozen cell stocks were stored for the long-term preservation of cells at low passage numbers. Fresh cells were thawed periodically or in the event a change in cell behaviour or morphology was noted to avoid experiments being influenced by potential changes in cell phenotype over time.

To create frozen stocks, cells were trypsinised, re-suspended in supplemented media, pelleted at 150 xg for 5 min and re-suspended in ice-cold FBS containing 10% dimethylsulfoxide (DMSO; J.T.Baker). Re-suspended cells at a concentration of 5-10 million cells/mL were transferred to cryovials (Greiner Bio-One, Kremsmuenster, Austria) and stored at -80°C for 1-3 days prior to long-term storage in liquid nitrogen.

Frozen stocks were revived by thawing rapidly to 37°C in a waterbath, re-suspending in supplemented media, pelleting the cells (to remove residual DMSO), re-suspending again in media, and transferring to a culture flask for maintenance. Media was changed and selective antibiotics added approximately 24 h later.

**Generation of stable cell lines**

All the transfected cell lines listed in Table 2.2 were transformed such that the introduced receptor was expressed stably over time. DNA was linearised with Scal restriction enzyme (Roche) to facilitate genomic DNA incorporation and transfected with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s recommendations. Briefly, 2 µL Lipofectamine™ 2000 per
100 uL Opti-MEM® (Invitrogen) was mixed and incubated at room temperature (RT) for 5 min. 8 ng/µL DNA was mixed with Opti-MEM®. The Lipofectamine™ 2000 and DNA mixes were then combined in equal volumes and incubated at RT for 20 min prior to 100 µL being added dropwise to cells that had been seeded (see below) in a 24-well plate and reached 90-100% confluency. 24 h post-transfection, cells were transferred to a 6-well plate. Another 24 h later selection antibiotics were added (350 µg/mL Zeocin™). In each transfection a no-DNA control was included so that death of cells not expressing the transfected construct could be monitored.

Following the death of the control cells (after approximately 2 weeks of maintenance in selection media), transfected cells were plated sparsely in a 6-well plate and allowed to grow in clonal colonies. Cells were labelled with primary and secondary antibodies to detect surface receptor (see below), and observed under a fluorescent microscope. Colonies expressing detectable levels of receptor were gently transferred into new culture dishes. As picking could not be performed under sterile conditions, cells were cultured with Penicillin-Streptomycin (Invitrogen) for 1 week to prophylactically prevent bacterial contamination. Surface and total receptor expression, and the clonal nature of the resultant cell lines were subsequently confirmed with standard immunocytochemistry.

**Cell plating for experiments**

Cells were seeded at an appropriate density to reach 70-80% confluence by the end of the experiment and allowed to recover overnight. Following trypsinisation (as above), a small aliquot of cells diluted in trypan blue (0.4%; Invitrogen) was counted in a haemocytometer. Cells were diluted appropriately in supplemented media and dispensed into a culture vessel with regular agitation to promote even distribution of cells between wells. For HEK cells a plating density of 26,000 to 30,000 cells per well in a 96-well plate would produce a confluency of 70-80% 24 h after seeding.
To aid cell adherence for trafficking or immunocytochemistry experiments, culture vessels were pre-treated with Poly-L-Lysine (0.2 mg/mL in PBS, Sigma-Aldrich) prior to cell seeding. Vessels were instead treated with Poly-D-Lysine (0.05 mg/mL in PBS, Sigma-Aldrich) if multi-chamber glass culture slides were used (for confocal imaging; BD Biosciences) or for experiments in which cells were to be treated with trypsin. Poly-L or Poly-D-lysine was incubated on the plastic or glass at 37°C for at least one hour, following which the plate was rinsed once with PBS.

**Trafficking assays**

Due to their lipophilicity, cannabinoids exhibit a tendency to be adsorbed by similarly hydrophobic surfaces, such as plastic, and have affinity for a number of the components in full-serum. Therefore, to promote the maintenance of cannabinoid drugs in solution, vessels used to dilute or dispense drugs were silanised prior to use (Coatasil, Ajax Finechem, Sydney, NSW) and drugs were diluted in serum-free media (SFM; DMEM or DMEM-F12 with 5 mg/mL BSA, ICPbio, Auckland, NZ; see also Hillard et al., 1995).

Prior to the start of each assay, cells were equilibrated in SFM for 15 min. The details of drug stimulations are noted in the text and were performed at 37°C unless otherwise stated. Table 2.3 lists the drugs and chemicals utilised. At the conclusion of drug stimulation, plates were placed on ice to prevent any further receptor trafficking, processed for immunocytochemistry as appropriate, and fixed (4% paraformaldehyde in 0.1 M phosphate buffer [PFA], 10 min at RT, followed by three PBS washes).

Vehicle and washing controls were included with each experiment. Final ethanol concentrations did not exceed 0.1%. A minimum of three, but regularly 4-5 replicate wells were included in each quantified experiment. The positioning of timepoints or drug conditions in 96-well plates was randomised for each experiment. In order to avoid effects of uneven evaporation from the edges of culture plates, the outside wells were filled with “sacrificial”
media and not assayed in the experiment. Except when rapid cooling was intended (at the end of an experiment and/or for immunocytochemistry protocols), cells were kept at a constant temperature by incubating in a 37°C incubator during long stimulations. When cells were removed from the incubator to add drugs, plates were placed on a polystyrene surface to prevent conduction of heat from the bottom of the plate. Experiments requiring frequent drug treatments over period of 30 min or less (internalisation) were performed with the cell culture plate sitting on the surface of 37°C waterbath to maintain a constant temperature.

If intracellular receptors were to be assayed in the experiment, prior to fixation or immunocytochemistry, cells were incubated with 0.05% trypsin-EDTA for 1 min at RT. Control cells for comparison were incubated with 0.2 g/L EDTA•4Na alone. Activity of the trypsin enzyme was halted by adding an equal volume of full-serum media or 2 mg/mL trypsin inhibitor from soybean (TIS; Sigma-Aldrich). Optimisation of this method is described in chapter three (pg. 64).
### Table 2.3 Drugs and chemicals utilised

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Type / function</th>
<th>Stock concentration and vehicle</th>
<th>Storage conditions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HU 210 (HU)</td>
<td>Cannabinoid agonant</td>
<td>10 mM, 100% ethanol</td>
<td>-80°C, or -20°C for up to 1 month</td>
<td>Tocris Bioscience, Ellisville, MO</td>
</tr>
<tr>
<td>WIN 55212-2 (WIN)</td>
<td>Cannabinoid agonant</td>
<td>20 mM, 100% ethanol</td>
<td>-80°C, or -20°C for up to 1 month</td>
<td>Tocris Bioscience</td>
</tr>
<tr>
<td>SR 141716A (SR)</td>
<td>CB₁ inverse-agonant</td>
<td>10 mM, 100% ethanol</td>
<td>-80°C, or -20°C for up to 1 month</td>
<td>National Institute on Drug Abuse, Rockville, MD</td>
</tr>
<tr>
<td>Dopamine hydrochloride</td>
<td>Dopamine agonant</td>
<td>100 mM, water</td>
<td>Prepared fresh for each experiment</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>SCH 23390</td>
<td>D₁ antagonist</td>
<td>10 mM, 100% ethanol</td>
<td>-80°C, or -20°C for up to 6 months</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Monensin sodium salt</td>
<td>Inhibitor of vesicle transport to plasma membrane¹</td>
<td>10 mM, 100% ethanol</td>
<td>-20°C for up to 6 months</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Cycloheximide (CHX)</td>
<td>Protein synthesis inhibitor²</td>
<td>50 g/L, 100% ethanol</td>
<td>-20°C for up to 6 months</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Concanavalin A (ConA)</td>
<td>Plant lectin, endocytosis inhibitor³</td>
<td>0.5 mM, 1 M NaCl</td>
<td>-20°C for up to 6 months</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

¹ (Mollenhauer et al., 1990); ² (Godchaux et al., 1967); ³ (Sato et al., 1976).
**Immunocytochemistry**

The antibodies utilised are listed in Table 2.4. Antibody testing and optimisation of labelling conditions is detailed in chapter three. Three principal immunocytochemistry protocols were applied. These were to detect: surface receptors prior to drug treatment (“live antibody feeding”), surface receptors at the conclusion of drug treatment, or total receptors (“post-fixation labelling”).

When surface receptors were to be labelled with primary antibody prior to drug treatment, cells were incubated for 30 min (unless stated otherwise) at 37°C with antibody diluted in SFM and washed twice with SFM prior to the addition of drugs. For detection of net surface receptor following drug treatment, cells were cooled rapidly on ice then incubated for 30 min at RT with primary antibody diluted in SFM and washed twice with SFM prior to subsequent immunocytochemistry or fixing. This protocol was also utilised to detect surface-localised primary antibody that had been applied prior to drug treatment; in this case secondary antibody was incubated with cells.

For experiments assaying total cellular receptor or protein, fixed cells were incubated with primary antibody diluted in immunobuffer (PBS with 1% normal goat serum, Invitrogen, and 0.4 mg/mL Merthiolate, Merck, Darmstadt, Germany) with 0.2% Triton X-100 for 3 h at RT or 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 PFA-fixed cells were treated with 90% methanol for 10 min at -20°C.

Secondary antibodies were incubated under the same conditions as for post-fixation primary antibody; diluted in immunobuffer, incubated at RT for 3 h or at 4°C overnight and washed once. Subsequently, cell nuclei were stained with Hoechst 33258 (8 μg/mL in PBS-T, 10 min at RT, Invitrogen) and washed twice again with PBS-T. 96-well plates for quantification were stored with 50 μL PBS-T with 0.4 mg/mL Merthiolate per well. Glass slides were mounted in AF-1 antifadant (Citifluor, Leicester, UK) with a #1.5 coverslip.
<table>
<thead>
<tr>
<th>Annotation in text</th>
<th>Epitope raised against</th>
<th>Species raised in</th>
<th>Dilution factors: live / post-fixation labelling</th>
<th>Cat. number, supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>HA</td>
<td>Mouse</td>
<td>1:500 / 1:1000</td>
<td>MMS-101, Covance, Princeton, NJ</td>
</tr>
<tr>
<td>L15</td>
<td>CB₁, 15 residues at extreme C-terminus</td>
<td>Rabbit</td>
<td>NA / 1:1000</td>
<td>NA, Prof. Ken Mackie¹</td>
</tr>
<tr>
<td>L73</td>
<td>CB₁, 73 residues at extreme C-terminus</td>
<td>Goat</td>
<td>NA / 1:25,000</td>
<td>NA, Prof. Ken Mackie²</td>
</tr>
<tr>
<td>ABR</td>
<td>CB₁, residues 1-77 at N-terminus</td>
<td>Rabbit</td>
<td>1:500 / 1:1000</td>
<td>PA1-745, Affinity BioReagents³</td>
</tr>
<tr>
<td>BioSource</td>
<td>CB₁, unspecified region in N-terminus</td>
<td>Rabbit</td>
<td>1:100 / 1:200</td>
<td>44-310, BioSource⁴</td>
</tr>
<tr>
<td>Cayman</td>
<td>CB₁, residues 1-14 at N-terminus</td>
<td>Rabbit</td>
<td>1:500 / 1:1000</td>
<td>101500, Cayman, Ann Arbor, MI</td>
</tr>
<tr>
<td>Mackie-NT</td>
<td>CB₁, residues 1-77 at N-terminus</td>
<td>Rabbit</td>
<td>1:250 / 1:500</td>
<td>NA, Prof. Ken Mackie⁵</td>
</tr>
<tr>
<td>Sigma</td>
<td>CB₁, residues 1-99 at N-terminus</td>
<td>Rabbit</td>
<td>1:500 / 1:1000</td>
<td>C1108, Sigma-Aldrich</td>
</tr>
<tr>
<td>Gα subunits</td>
<td>Gα i1 residues 159-168, or Gα i3 and o residues 345-354</td>
<td>Rabbit</td>
<td>NA / 1:500</td>
<td>371720 &amp; 371726, Calbiochem⁶</td>
</tr>
<tr>
<td>Alexa Fluor® 488 and 594</td>
<td>Mouse, Rabbit or Goat IgG</td>
<td>Goat or Donkey*</td>
<td>1:300 / 1:400</td>
<td>Various*, Invitrogen.</td>
</tr>
</tbody>
</table>

**Table 2.4 Antibodies utilised**

*For the majority of assays secondary antibodies raised in goat were utilised, with the exception of single and double-labelling experiments with the L73 antibody for which secondary antibodies raised in donkey were used. Catalogue numbers were: A11029, A11032, A11034, A11037, A21202 and A11058. ¹(Nyiri et al., 2005; Eggan and Lewis, 2007); ²(Bodor et al., 2005); ³Subsidiary of Thermo Scientific; ⁴Subsidiary of Invitrogen; ⁵(Tsou et al., 1998; Twitchell et al., 1997), ⁶Subsidiary of Merck.
**Imaging and quantification**

The majority of imaging and quantification was performed with the Discovery-1™ automated fluorescent microscope (Molecular Devices, Sunnyvale, CA) and Metamorph® image analysis software (v. 6.2r6, Molecular Devices). Details of the system and quantification method are provided in chapter three. The majority of widefield images presented were acquired with the Discovery-1™ microscope using a 40 x objective lens. A few widefield images, as indicated, were obtained with a 60 x objective lens (Nikon Plan Apo, NA 1.4) and colour digital camera on an upright fluorescence microscope (Nikon, Tokyo, Japan). Confocal images were obtained on a Leica TCS SP2 system with 63x objective lens (Leica HCX PL APO, NA 1.32), Airy 1 pinhole, and line averaging (8). Images for presentation were edited with Photoshop CS4 Extended (v. 11.0; Adobe, San Jose, CA). Images underwent only linear adjustments to brightness and contrast, and multi-colour images were produced by overlaying the single-colour images with the 'difference’ filter layer mode. Presented images are representative of three to four independent experiments.

**cAMP assays**

Inhibition of cAMP accumulation was assessed with a competition binding assay. Briefly, having been prepared as for trafficking experiments (above), cells were incubated with 0.5 mM isobutyl methylxanthine (IBMX; Sigma-Aldrich) in SFM for 15 min, then cannabinoid drugs with 50 µM forskolin (Tocris Bioscience) and IBMX in SFM for 15 min, all at 37°C. This forskolin concentration produced approximately 80% of the maximum possible cAMP response in the rCB₁ HEK cell line when applied alone. At the conclusion of drug stimulation, media was aspirated and ice-cold 100% ethanol added. Assay plates were incubated at -20°C for at least 10 min, then ethanol was evaporated off at RT. Samples were re-suspended in assay buffer (20 mM HEPES, 5 mM EDTA, pH 7.5) and incubated at RT for 5 min, then transferred to a round-bottom 96-well plate. ³H-cAMP (Amersham, Chalfont St. Giles, UK) and cAMP-dependent protein kinase A (Sigma-Aldrich) were added to final concentrations of 0.5 µCi/mL and 0.01% w/v respectively, and incubated for 2 h to overnight at 4°C. Subsequently, activated
charcoal was added (100-400 mesh, final concentration 1.7% w/v with 0.07% BSA w/w in assay buffer; Sigma-Aldrich) and pelleted by centrifugation at 3,000 x g for 5 min at 4°C. Supernatants were taken into scintillation counter-compatible plates and scintillant added (Starscint, Packard Bioscience, Meriden, CT). Emitted light was quantified with a Wallac 1450 Microbeta Jet Trilux scintillation counter (Perkin Elmer, Wellesley, MA). Data was normalised such that 100% was equal to cAMP in cells treated with forskolin only, while 0% was equal to cAMP in cells treated with vehicle only.

**Western blotting**

Cell pellets were prepared by washing with PBS, incubating for 2-3 min with 0.2 g/L EDTA•4Na or trypsin (as appropriate), adding an equal volume of media and centrifuging at 500 x 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]). Samples were incubated on ice for 30 min then centrifuged at 14,000 x g for 10 min. For peptide-N-glycosidase F (PNGase F; Sigma-Aldrich) treatment, samples were subsequently incubated at 65°C for 5 min with 52.6 mM β-mercaptoethanol, cooled, and incubated with ~0.04U enzyme per µg protein at 37°C for 18 h.

Samples were diluted 1:2 in 2x 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). The SeeBlue Plus2 pre-stained standard (Invitrogen) was run alongside samples.

Membranes were incubated with 5% non-fat milk (NFM) in Tris-buffered saline with 0.05% Tween (TBS-T; 30 min at RT), then primary antibody (anti-HA diluted 1:5000) in 1% NFM/TBS-T overnight at 4°C. Membranes were washed (3 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 3 further washes, membranes were incubated with ECL-plus (GE Healthcare) for 5 min and the chemiluminescent signal detected with autoradiographic film (GE Healthcare).

Films were digitally scanned at 600dpi for presentation (HP Scanjet 3770, Hewlett Packard, Palo Alto, CA). Blot images are representative of three to four independent experiments.

**Data presentation and statistics**

Data is presented as the mean ± standard error of the mean from three to four independent experiments. 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. The full details of statistical tests applied are provided in the appendix (pg. 171-179). p-value significance levels are represented graphically as follows: *, p < 0.05; **, p < 0.005; ***, p < 0.001.
CHAPTER THREE

QUANTITATIVE ASSAY DEVELOPMENT

INTRODUCTION

A variety of techniques have been used successfully to study receptor trafficking. However when considering the objectives to be addressed in this thesis, it is clear that the limitations of the majority of these approaches would severely hinder progress of the research.

Ligand binding, for example, provides only an indirect measure of receptor numbers in ligand-accessible compartments. Results may be dependent on the activity state of the receptors, which is likely to be influenced by both receptor trafficking and signalling, and the particular radioligand used. The interpretation of data from binding assays would be further complicated in this study because the majority of cannabinoid ligands are lipophilic. These tend to exhibit high levels of non-specific binding and can permeate cell membranes. However the extent to which this permeation occurs (and consequently the accessibility of intracellular receptors) is not fully understood, therefore making quantitation of surface versus total CB₁ problematic in this context.

Immunocytochemistry-based approaches facilitate direct detection of receptors. Analysis of confocal micrographs by ratiometry allows accurate delineation of intracellular compartments, however is particularly laborious (resulting in small sample sizes) and can be vulnerable to human bias. While flow cytometry offers accurate quantification and exquisite sensitivity, sample preparation, processing and quantification can be time consuming. Cell-based ELISA
is a quantitative high-throughput method that was considered for this study, however normalisation for differences in cell density between conditions is not necessarily reliable (Glass lab, unpublished observation), and ELISA does not readily allow for quantification and visualisation of receptors in the same process, which was a desired quality in this study.

High-throughput microscopy-based techniques address most of the limitations of these assays. Data acquisition and analysis is rapid, unbiased, and largely automated. The acquisition of images for analysis allows researchers the option to qualitatively confirm results by eye and observe changes in receptor localisation that would not necessarily be noticed during quantification. One well established high-throughput assay for measuring receptor internalisation is based on the quantification of clusters of endocytic vesicles that appear in the cytoplasm as receptors internalise from the cell surface, usually as a result of ligand-induced receptor activation. When identified with fluorescent probes, these clusters appear as bright puncta or “granules”, the quantity of which has been demonstrated to correlate with receptor endocytosis (Conway et al., 1999; Lee et al., 2006). A limitation of this approach is that its use is restricted to the detection of early endocytosis, and it does not lend itself well to recycling or degradation assays as a change in granule number could indicate either process. The novel method optimised in this study also utilises fluorescent probes, but relies on selective detection of surface, total or intracellular receptors with primary and secondary antibodies. Subsequent detection of fluorescent signal arising from the sample, normalised to the number of cells in the field of view, allows changes in receptor expression and localisation following pharmacological treatment to be measured.

The integrity of this method is therefore crucially dependent upon the quality - that is, efficacy and specificity - of antibodies used. For the most part, the experiments in this study were performed on heterologously expressed HA-tagged receptor. As epitope tags are utilised for research in many fields, a range of reliable compatible reagents are commercially available. The presence of the tag at the amino (extracellular) terminus allowed for detection of either
total or surface receptor. However, experiments on native CB$_1$ were also planned. Therefore antibodies that would reliably detect CB$_1$, and preferably that were directed against the N-terminus for selective detection of surface receptors, were also required. While the production of quality antibodies can be challenging, several CB$_1$ antibodies targeting different regions of the receptor have been developed by research groups and a number are available through commercial sources.

This chapter will demonstrate the testing and selection of antibodies for use in subsequent experiments and the development of a novel high-throughput method for studying receptor trafficking. The method, based on widefield fluorescent imaging (automated with a Discovery-1™ microscope) and measurement of the signal intensity per cell (MetaMorph® analysis software), is validated by measuring the timecourse of CB$_1$ internalisation in comparison with results obtained with “Granularity”, an in-built MetaMorph® assay analysis. In addition, the development of a method for selective detection of intracellular receptors is described and drug concentrations for use in later experiments are selected.

**Methods**

General materials and methods used are described in chapter two. Experiments in this chapter were conducted on the HA-rCB$_1$ HEK cell line and, unless noted, utilised anti-HA primary antibody. For detection of receptors originating at the surface or net surface receptor, primary antibody was applied to live cells prior-to or following drug stimulation (respectively), as indicated. Secondary antibody was then incubated under various conditions depending on the exact assay; with live cells at RT to detect remaining surface primary antibody-labelled CB$_1$, or post-fixation under permeabilising conditions to detect internalised antibody-labelled CB$_1$ or net surface CB$_1$ (when primary antibody had been applied at the end of the experiment). Total CB$_1$ was detected by applying both primary and secondary antibodies following fixation under permeabilising conditions.
Colocalisation

In order to assess the degree of colocalisation in dual immunocytochemistry experiments, cytofluorograms were produced and Pearson's coefficients calculated with the “Colocalization Finder” plug-in for ImageJ (C Laummonerie & J Mutterer, http://rsb.info.nih.gov/ij/plugins/colocalization-finder.html). The cytofluorograms display the correlation of pixel intensities between the two images; higher frequencies of pixel-intensity combinations are represented as hot colours. Prior to calculation of the Pearson's coefficient, images were converted to 8-bit (greyscale) and thresholded at the boundary of “real” and background staining to exclude areas of the image not containing cells; the threshold levels are represented as dotted lines on the cytofluorogram plots.

Image acquisition with Discovery-1™

The Discovery-1™ high-throughput imaging platform incorporates an inverted widefield microscope with a motorised stage (Prior Scientific Instruments, Cambridge, UK), z-axis driver (with step resolution of up to 1 μm) and a monochrome peltier-cooled 12-bit digital CCD camera (Hamamatsu Photonics, Japan). Fluorescent specimens are illuminated with a 175W xenon lamp. 10x and 40x objective lenses were used (Nikon Plan Fluor, NA 0.3 and 0.6 respectively).

Four images from adjacent sites in the centre of each well of a 96-well plate were acquired for Hoechst, Alexa Fluor® 488 and/or Alexa Fluor® 594 staining with the “DAPI” (exposure time 500-1500ms, typically ~800ms), “FITC” (exposure time 800-3000ms, typically ~1500ms) and “TRED” filters (exposure time 800-3000ms, typically ~1500ms) respectively. EGFP images were also acquired with the “FITC” filter set (exposure time 100-1500ms, typically ~300ms). Filter settings for excitation and emission of different fluorophores are provided in Table 3.1 (pg. 46). To ensure that images were not saturated but that sensitivity was maintained, exposure times for each set of experiments that had undergone immunocytochemistry simultaneously were set empirically on wells expected to have the highest levels of staining.
Samples underwent autofocusing with the in-built “low signal” focusing algorithm, whereby low exposure, bin-averaged (4x4) images are rapidly assessed to select the optimal focal plane. The focal plane was established with a “wide” focus range (70 µm) on the first site per well for DAPI, and refined with “fine” focus (35 µm) for subsequent wavelengths and sites. Focusing accuracy (z-axis stepping) was set to 4 µm at 10x and 1 µm at 40x.

Owing to the relatively high image resolution required to successfully assess Granularity (see below), internalisation experiments comparing the results obtained with Granularity to our novel approach were acquired with the 40x objective and 2x2 binning (~100 cells/image). As the new method described here does not require high magnification images, subsequent experiments were acquired with the 10x objective. At this magnification within-experiment data variability was reduced, most likely due to the larger sampling of cells (~1000 cells/image).

The 1344x1054 pixel 16-bit tiff images (with grey levels in the 12-bit range due to the camera limitations, ie. assigned values of 0-4095) were quantified using MetaMorph® software as detailed below. Any images affected by brightly fluorescent debris or that were not correctly focused were excluded from subsequent analysis.
<table>
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<th>Dichroic mirror (nm)</th>
<th>Emission range (nm)</th>
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Table 3.1 Discovery-1™ filter and dichroic mirror settings with associated fluorophore excitation and emission properties
Assessment of receptor internalisation by Granularity

The Granularity function utilised is available as a “drop-in” assay for MetaMorph®. This assay identifies granules as distinct focal regions that have pronounced differences in intensity from the immediately surrounding pixels. User-set parameters define the approximate minimum and maximum width of granules and the typical difference in intensity of granules compared to background, which are used by the software to identify, count and assess the size and intensity of granules in an image.

The “Count Nuclei” drop-in assay was utilised to assess the number of nuclei (and therefore cells) in the associated Hoechst staining images. It is necessary to optimise user-defined parameters (minimum and maximum nuclei width and intensity above local background) for particular cell types and general staining intensities; however the same settings could usually be applied across different experiments with the same cell type. The count nuclei assay was found to be relatively robust to changes in cell density, however cell counts tended to be underestimated if cells were over-confluent. The number of granules per image was divided by the nuclei count to calculate the average number of granules per cell in each image.

At the facility used, MetaMorph® is run on an Intel Pentium 4 CPU 2.60GHz computer with 2.00GB RAM. The Granularity assay took approximately 0.75 sec/image and the Count Nuclei assay took approximately 2.5 sec/image. Processing times are influenced by exact computer specifications.

Assessment of receptor expression by Total Grey Value per Cell

The method of image analysis developed to address the research questions in this thesis is based on straightforward image segmentation by thresholding and subsequent assessment of the total signal intensity arising from each cell in an image, on average. This approach is henceforth referred to as “Total Grey Value per Cell” (TGVC).
To undertake analysis with this method, a user-defined threshold level is set whereby pixels with grey values below this level will not be included in the analysis. MetaMorph® applies this threshold to each of the images stained for the protein of interest and calculates outputs including the integrated intensity (total grey value), and total pixels within the threshold range. Lower cut-off threshold levels were set at a value similar to the weakest cellular staining visible. Data collection was automated with a journal entitled “Thresholded Average Intensity” written by M. Dragunow. The Count Nuclei assay was used to determine the number of cells in each image (as described above).

The fluorescent signal arising from cells is the sum of real staining (from secondary antibody or EGFP fluorescence) and background staining (from non-specific antibody labelling, autofluorescence of the sample or plate, and ambient light). Although segmenting the image with a threshold excludes background staining in areas where there are no cells, the background staining present in areas with cells will contribute to the integrated grey value. This was corrected for by assuming the threshold level set approximately equalled background staining and subtracted this grey value multiplied by the number of pixels in the thresholded area from the integrated grey value. This background-corrected value for integrated grey value was finally divided by the number of nuclei counted in the image.

Thereby, the final calculation used to assess TGVC is:

\[
TGVC = \frac{\text{Total Grey Value} - (\text{Pixels In Threshold Range} \times \text{Threshold Level})}{\text{Cell Count}}
\]

At the facility used, the Thresholded Average Intensity journal took approximately 0.06 to 0.08 sec/image and the Count Nuclei assay took approximately 2.5 to 5 sec/image to analyse 400x and 100x images, respectively. Processing times are influenced by exact computer specifications.
RESULTS

Selection of antibodies and verification of specificity

As already mentioned, access to antibodies that would specifically detect HA-tagged receptors or native CB₁ in immunocytochemistry would be crucial for the quantification of trafficking experiments with the proposed approach. An anti-HA antibody and a range of anti-CB₁ antibodies were therefore tested for their efficacy and specificity. The antibody details are provided in Table 2.4, pg. 37.

Initially, the anti-HA antibody was tested. Incubation of antibody with live cells for 30 min at 37°C produced continuous staining at the surface of cells, evident because of clear lines of staining in between nuclei (Figure 3.1A “HA”) and later confirmed by confocal microscopy (eg. Figure 3.4). When CB₁ agonist was applied following antibody labelling (and secondary antibody applied to permeabilised cells post-fixation), staining was re-distributed to the cytoplasm and appeared as bright intracellular puncta (Figure 3.1B “HA, Agonist 15 min”) which was consistent with CB₁ internalisation (Hsieh et al., 1999). Incubation of live HEK wt cells (that did not express CB₁) produced no detectable staining (Figure 3.1B “HA, No-CB₁ Control”). Application of anti-HA antibody to fixed and permeabilised cells also produced an expected pattern of staining, with significant proportions of receptor both at the cell surface and in the cytoplasm (Figure 3.2 “anti-HA”, green). The latter portion is commonly referred to as the “intracellular pool” (eg. Leterrier et al., 2004). Again, no detectable staining was detected on HEK wt cells when the antibody was applied under the same conditions (Figure 3.3 “HA”).

Subsequently, the antibodies designed to recognise epitopes in the CB₁ N-terminus were assessed for their ability to detect surface CB₁. Of the group, only the Mackie-NT antibody produced a pattern of staining reminiscent of surface receptors; the commercially available antibodies each produced very low-level staining that was sometimes punctate (eg. Sigma) but otherwise hazy and non-descript (Figure 3.1A). The Mackie-NT antibody was tested for
detection of CB$_1$ internalisation and lack of staining on HEK wt cells, and produced results similar to those for anti-HA (Figure 3.1B “Mackie-NT”).

The full range of anti-CB$_1$ antibodies were next tested for their efficacy in detecting total CB$_1$. Although an N-terminally directed antibody would be most useful for the planned receptor trafficking experiments because surface CB$_1$ can be detected selectively, a C-terminally directed antibody could also be useful for total receptor studies. As the anti-HA antibody appeared to be specific for HA-CB$_1$, staining was compared with the CB$_1$ antibodies in double-labelling permeabilised immunocytochemistry experiments (Figure 3.2). The C-terminal L15 and L73, and Mackie-NT antibodies both produced what appeared to be specific CB$_1$ staining that was very similar in appearance to that for anti-HA. Analysis of these images with cytofluorograms and Pearson’s coefficients also indicated the staining was well colocalised. The remaining CB$_1$ antibodies exhibited varying patterns of staining, both cytoplasmic and nuclear, that was not reminiscent of the anti-HA staining in the same cells. The cytofluorogram and Pearson’s coefficient analyses indicated there was poor correlation of pixel intensities between anti-HA and these CB$_1$ antibodies. The L15 and Mackie-NT antibodies exhibited minimal non-specific staining on HEK wt cells (Figure 3.3).

Sequential immunocytochemistry was performed both with the anti-HA incubation before and after the anti-CB$_1$ incubation (to reduce the likelihood that the presence of anti-HA would inhibit N-terminally directed antibody binding), and CB$_1$ antibodies were also tested on their own, with similar results to those indicated in Figure 3.2. As it is feasible that the presence of the HA tag on CB$_1$ could inhibit antibody binding, it would have been preferable to test the antibodies on cells that endogenously express CB$_1$. However, the cell line available at the time of the study (Neuro2a, utilised in later experiments) exhibited very low levels of CB$_1$ expression making it difficult to compare staining patterns between antibodies, and there was no appropriate negative control available (ie. a cell line that did not express CB$_1$ but expressed the full complement of other proteins expressed in the Neuro2a line).
These results therefore suggest that the commercially available anti-CB₁ antibodies tested have poor specificity for their intended target by immunocytochemistry. However, the suitability of anti-HA for detecting HA tagged receptor and the L15, L73 and Mackie-NT antibodies for detecting CB₁ was confirmed. In the subsequent experiments described in this thesis, the anti-HA antibody was utilised whenever HA-CB₁ was studied, while the Mackie-NT and L15 antibodies were used to detect surface and total CB₁ (respectively) in Neuro-2a cells.
Figure 3.1 Comparison of antibodies for surface CB₁ immunocytochemistry

(A) Detection of surface HA-CB₁ by live-cell labelling. Abundant surface CB₁ is detected with the HA and Mackie-NT antibodies. The lack of staining detected with the ABR, BioSource, Cayman and Sigma N-terminal antibodies is also demonstrated. (B) HA or Mackie-NT antibody localisation following live labelling of HA-CB₁ and incubation with vehicle or agonist (WIN 1 µM) for 15 min. Also shown is live antibody labelling on un-transfected cells (“No-CB₁ Control”). Bars, 15 µm. Images were obtained on a Nikon upright microscope with the same settings and exposure times for comparative images within antibodies.
A

HA ABR BioSource

Cayman Mackie-NT Sigma

Vehicle 15 min Agonist 15 min No-CB₁ Control

B

HA

Mackie NTer

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Figure 3.2  Comparison of antibodies for total CB\textsubscript{1} immunocytochemistry

Detection of HA-CB\textsubscript{1} in fixed and permeabilised cells with double immunocytochemistry using anti-HA (green) in combination with anti-CB\textsubscript{1} antibodies (red). Merged images show relative co-detection of receptors. Images were obtained on a Nikon upright microscope. Bar, 10 μM. The cytofluorograms ('Plot') display the correlation of pixel intensities between the HA and CB\textsubscript{1} antibody images. X and Y axes ticks correspond to 50 grey levels. Pearson’s coefficients, displayed in the top right-hand corner, were calculated on pixels above a grey level deemed to represent the limit between “real” and background staining (threshold level represented by dotted line).
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<table>
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Figure 3.3 Test for non-specific staining of selected antibodies on fixed and permeabilised cells not expressing CB₁

In order to detect non-specific staining, the HA, L15 and Mackie-NT antibodies were incubated with fixed and permeabilised un-transfected HEK wt cells, which do not express CB₁ endogenously. Immunocytochemistry and imaging were performed at the same time and under the same conditions as the corresponding positive staining images in Figure 3.2. Images were obtained on Nikon upright microscope. Bar, 10 μM.
Selection of conditions for antibody recognition of surface receptors

As many receptor trafficking studies focus on protein transport to and from the plasma membrane, and indeed this formed the core of the experiments planned in this study, it is particularly important to be able to reliably measure surface receptor expression under a variety of conditions. As indicated in Figure 3.1B, incubating primary antibody with live cells at 37°C prior to drug treatment successfully labelled surface receptors and did not appear to inhibit their internalisation. While this approach would be useful in certain experiments, such as those in which the fate of receptors originating at the surface prior to drug treatment is of interest, it would not be appropriate for experiments where it would be desirable to measure net surface receptor expression at the conclusion of drug stimulation.

This alternative experimental design can simply be addressed by incubating cells with primary antibody at the end of the experiment. However, the conditions under which this incubation is performed are crucially important. Firstly, it is imperative that surface receptor trafficking is halted, as continued trafficking resulting from the pharmacological treatment would effectively result in a different timepoint being measured than intended. Secondly, the potential effect of whatever method is used to halt trafficking on antibody labelling must be considered. Note that applying primary antibody post-fixation under non-permeabilising conditions was piloted, however this approach was deemed unreliable as a small degree of cell permeabilisation was observed, perhaps arising during fixation or due to detergent contamination of lab glassware.

It has previously been reported that surface trafficking is inhibited at reduced temperatures (4-16°C; von Zastrow and Kobilka, 1994). Here, antibody live labelling was compared at three incubation temperatures; 37°C, RT and 4°C (preceded by ~1 min on ice for both RT and 4°C to rapidly cool cell media). RT was approximately 21-23°C, and consistent day-to-day due to ambient temperature control with air conditioning. In order to mimic a typical experimental design, cells were co-incubated with antibody and either vehicle or CB₁ agonist for 30 min. Secondary antibody was applied post-fixation under permeabilising conditions to detect both
surface and intracellular antibody-bound CB₁. Imaging of cells by confocal microscopy revealed that, while the most intense receptor staining was obtained at 37°C, a significant degree of both constitutive (Figure 3.4A) and agonist-induced (Figure 3.4B) CB₁ internalisation proceeded, as is evidenced by the presence of intracellular punctate staining. However, constitutive and agonist-induced trafficking appeared to be completely inhibited at both RT and 4°C (Figure 3.4). Interestingly, as the antibody incubation temperature was reduced, the level of receptor-antibody staining also decreased markedly. It may have been possible to increase the staining intensity at the lower temperatures by incubating cells for longer time periods, however this would increase the risk that cell morphology or other characteristics might alter following the end of the experiment.

It therefore appeared that in terms of halting receptor traffic, incubation at both RT and 4°C would be suitable for detection of net surface receptors following drug treatment. However as a significantly higher level of staining was observed at RT, this condition was selected for use in subsequent experiments. Although some constitutive internalisation appeared to occur while incubating antibody with cells at 37°C, this approach was preferred for pre-drug treatment labelling so as to maintain cells in consistent conditions during experimentation and to allow for staggered labelling and stimulation designs within one assay plate.
Figure 3.4 Detection of surface CB₁ at different temperatures in the presence or absence of agonist

Confocal images of HA-CB₁ HEK cells incubated with HA primary antibody in the presence of vehicle (A) or HU 1 µM (B) for 30 min at the temperatures indicated. Secondary antibody was applied to permeabilised cells post-fixation. Bars, (A) 20 µm, (B) 10 µm.
CB₁ internalisation quantified with TGVC and Granularity

Having confirmed appropriate antibodies and receptor labelling conditions for use in this study, the proposed high-throughput quantitative method, TGVC, was validated by demonstrating a CB₁ internalisation timecourse and comparing the results with those obtained via a Granularity assay.

When running the Granularity assay, the initial selection of parameters (granule size and intensity) was highly subjective, however once decided upon only the intensity setting required optimisation between experiments. Variability in receptor staining within the cell membrane often resulted in the detection of false-positive granules (that is, granular staining not associated with internalised receptor), therefore the parameters selected resulted in a trade-off between detecting most of the “true” granules in agonist-treated conditions yet few “false” granules in the vehicle-treated control.

The TGVC analysis required image thresholding, which enables the user to segment regions of interest from background noise and pixels. Due to slight variations in staining intensity, it was not possible to apply the same threshold levels between experiments. This step was therefore necessarily subjective, however the boundary between “real” and background staining was readily discernible, and small variations in the selected threshold level only slightly influenced the magnitude of quantified effects. Therefore while absolute values were difficult to replicate, the relative trends seen were consistently reproducible between experiments.

Incubation of CB₁-expressing cells with cannabinoid agonist HU (100 nM) resulted in receptor internalisation, as was evidenced by an increase in the number of intracellular granules per cell (Figure 3.5 A-C), and a loss of cell-surface fluorescence over time (Figure 3.5 D-F). Both approaches indicated that internalisation proceeded rapidly, reaching a maximum extent within 20 min, and were able to be modelled with exponentially derived curves. Interestingly, the half-lives indicate that internalisation proceeds more rapidly if assessed by Granularity (2.31 ±
0.036 min) than if measured with TGVC (3.35 ± 0.19 min; \( p = 0.043 \)). Data was highly reproducible between experiments for both approaches. These experiments were both performed with live primary antibody labelling prior to the addition of drug treatment and secondary antibody applied under permeabilising (Granularity) or non-permeabilising (TGVC) conditions. The timecourse of internalisation was also assessed by TGVC when primary antibody was incubated at RT subsequent to drug treatment (Figure 3.5 G). The TGVC-measured internalisation timecourses were not significantly different whether receptor labelling was performed prior-to or after the addition of drugs (\( p = 0.62 \)), indicating that the interaction of antibody with receptor did not influence agonist-driven endocytosis.

“Non-specific” staining, as indicated by primary and secondary antibody labelling on untransfected HEK cells, was quantified to represent 0.78 ± 0.41% of positive control rCB\(_1\) HEK cell staining (HA, Mackie-NT and L15 antibodies tested), suggesting that the threshold subtraction method used in the TGVC calculation produced a close approximation of true zero and would therefore quantify receptor expression changes within an appropriate window. The “non-specific” signal being marginally measurable also indicated that the limit of detection encompassed the full possible dynamic range for receptor expression, that is, the assay would have the potential to measure reductions in expression down to zero receptors (which would yield a marginally positive value).
Figure 3.5  CB₁ agonist-induced internalisation, quantified with TGVC and Granularity

Internalisation induced by incubation with HU 100 nM and assessed by Granularity (A-C) or TGVC (D-G). Primary antibody was incubated prior to starting drug treatment; secondary antibody was applied under permeabilising (Granularity, A-C) or non-permeabilising (TGVC, D-F) conditions. Internalisation is represented by an increase in the number of receptor-associated puncta per cell over time when quantified by Granularity (A), whereas the receptor staining for TGVC reveals a decrease in cell surface-associated fluorescent signal (indicating loss of cell surface receptor) over time (D). (B, E) Montages of images acquired with Discovery-1™ (images from each of four sites in one representative well for each timepoint, as indicated, at reduced resolution). Bars, 100 μm. (C, F) High resolution images of 0 or 25 min agonist stimulation demonstrate the aforementioned changes in receptor staining associated with CB₁ internalisation. Bars, 15 μm. (G) Internalisation, again induced with HU 100 nM and assessed by TGVC, but with the primary antibody applied at the conclusion of drug stimulation at RT.
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A

B

C

D

E

F

G
Development of method for selective detection of intracellular CB₁

One CB₁ characteristic anticipated to be of particular interest in this study is the presence of a large intracellular pool of receptors. Although two studies in particular have performed initial investigations into the functional role of the intracellular pool (Leterrier et al., 2004; Rozenfeld and Devi, 2008), the conclusions reached were based largely on indirect evidence or the quantification of very small numbers of cells by confocal micrograph ratiometry. Development of a method whereby intracellular CB₁ expression could be monitored separately from surface receptors without laborious image segmentation or cell fractionation would therefore represent a significant contribution to techniques for studying receptor trafficking and would certainly be useful in the present research. A straightforward immunocytochemical approach to achieve this would be to block surface antibody labelling, leaving only signal from intracellular receptors detected. Here, a method adapted from Czajkowski et al. (1989) and McIntosh et al. (1998) that exploits enzymatic digestion of extracellular protein is optimised for immunocytochemistry and analysis with the high throughput method previously described.

Trypsin is a proteolytic enzyme commonly used in tissue culture to suspend adherent cells and in mass spectrometry-based proteomics to fragment proteins. There are nine trypsin recognition sites in the human, rat and mouse CB₁ (long isoform) extracellular tails (carboxyl to lysine or arginine, Olsen et al., 2004), cleavage at any of which would be expected to separate the HA epitope and render CB₁ unrecognisable to the HA antibody. As trypsin can also digest cell adhesion molecules this potential technique required careful optimisation to ensure the cleavage of the CB₁ N-terminus without significant disruption of cell-substrate attachment. In pilot experiments it was noted that a greater number of cells were retained on the assay plate when the plastic was pre-treated with PDL as opposed to PLL (qualitative observation), likely because PDL is resistant to trypsic digestion whereas PLL is a ready substrate (Tsuyuki et al., 1956). Cells were therefore seeded on PDL-treated substrate for any experiments in which the trypsin method was used. 0.05% trypsin-EDTA was used throughout these experiments, as
previously (McIntosh et al., 1998). EDTA alone acted as the vehicle control and did not influence CB₁ expression or cell density (data not shown).

In order to optimise the time of trypsin exposure and to be useful in trafficking studies it would be necessary to be able to halt activity of the enzyme. It is well known that a serum component, α₁ antitrypsin, potently inhibits trypsin activity. FSM (DMEM with 10% FBS) was therefore tested for its ability to block trypsinisation, as was a trypsin inhibitor from soybean (TIS) at 2 mg/mL. These potential inhibitors were compared with SFM (DMEM with 5 mg/mL BSA) which was not expected to inhibit trypsin activity. Cells were washed twice with PBS, then incubated with trypsin mixed 1:1 with inhibitor for 2 min at RT. The trypsin was added to inhibitor and mixed immediately before dispensing on cells. As FSM was expected to be an effective trypsin inhibitor, an excess of full serum media was added to all conditions at the conclusion of the incubation. Cells were subsequently placed on ice, washed and incubated with primary antibody at RT to detect non-trypsinised surface CB₁. As demonstrated in Figure 3.6, although essentially no surface HA staining was retained with the SFM-trypsin mix, both FSM and TIS prevented trypsin-mediated cleavage as is evidenced by the lack of a significant change in staining compared to the vehicle control ($p = 0.92$ and 0.35 respectively). There was no significant reduction in cell number with any treatment ($p = 0.073$), although there was a trend toward cell loss with SFM (that is, no trypsin inhibition). As FSM trended toward being slightly more efficacious in blocking trypsin, both in terms of remaining surface HA-CB₁ and cell counts, FSM was used to halt trypsin treatments in the majority of subsequent experiments. Also, from this point SFM was used to wash cells prior to trypsinisation as it did not inhibit the reaction and would be consistent with the media used for drug incubations in trafficking experiments.

The timecourse of trypsin-mediated cleavage of HA-CB₁ was then investigated. Surface and total HA-CB₁ (not cleaved by trypsin) and cell counts were monitored for 0 to 15 min. As shown in Figure 3.6B, even after only 30 sec of trypsin treatment surface HA-CB₁ staining was
reduced to the limit of detection and total HA-CB₁ was reduced to ~40% of vehicle-treated cells, a proportion that approximately matched the size of the intracellular pool observed by eye in initial immunocytochemistry studies. The effect on anti-HA staining was similar at all timepoints, however cell numbers were markedly reduced at the 5, 10 and 15 min timepoints.

In order to ensure complete cleavage of extracellular HA epitopes from surface CB₁, but to avoid significant reductions in cell number during the treatment, a 1 min timepoint was selected for use in later experiments. Although a 30 sec incubation was considered, 1 min was found to be more practical when carrying out experiments on full 96-well plates, that is, trypsin was added at staggered (1 sec) intervals across the plate then 1 min later FSM was added 1:1 in the same spatial and temporal pattern to stop the reaction. The effect of trypsin on anti-HA staining is visualized in Figure 3.6C. Incubation with trypsin for 1 min at RT rendered surface HA-CB₁ (green) unrecognizable to anti-HA, leaving only intracellular receptor detected (red).
Figure 3.6 Demonstration and optimisation of trypsin treatment for selective detection of intracellular CB₁

(A) SFM, FSM or TIS were mixed 1:1 with trypsin immediately prior to incubating with cells for 2 min. Surface HA-CB₁ and cell counts were measured relative to vehicle-treated controls. (B) Timecourse of trypsin treatment and effect on surface and total HA-CB₁ as detected by anti-HA, and cell count. (C) Confocal micrographs of HEK HA-rCB₁ cells incubated with vehicle or trypsin for 1 min. HA primary then Alexa Fluor® 488-conjugated secondary antibodies were incubated with live cells to detect surface HA-CB₁ (green). Cells were then fixed and permeabilised, and incubated again with HA primary then Alexa Fluor® 594-conjugated secondary antibodies to detect total/intracellular HA-CB₁ (red). Bar, 10 µm.
Selection of appropriate drug concentrations for co-stimulation experiments

Pharmacological reagents such as inhibitors of internalisation, vesicle transport and protein synthesis are valuable tools in receptor trafficking studies. However, the interference of such drugs with fundamental cellular processes can alter cellular phenotypes and be deleterious to cell health. These potentially unpredictable changes may affect the pathways of interest via mechanisms unrelated to the intended pharmacological effect. To minimise this risk, these drugs should be used cautiously and care should be taken in selecting the concentrations and conditions under which such drugs are to be used.

The protein transport-modifying reagents selected for use in this study were: monensin, an inhibitor of vesicle acidification and thereby vesicle delivery to the cell surface (Mollenhauer et al., 1990); CHX, an inhibitor of translational elongation and thereby protein synthesis (Godchaux et al., 1967); and ConA, an inhibitor of internalisation that likely acts by cross-linking extracellular glycosyl residues (Sato et al., 1976). Another reagent of interest was SR, a CB₁ inverse-agonist.

Changes in surface and/or total CB₁ were utilised to select appropriate drug concentrations. Based on the drugs’ anticipated cellular effects and previous studies with CB₁, it was expected that monensin would reduce surface CB₁ by blocking delivery of newly synthesised and recycling receptors to the plasma membrane, CHX would reduce total CB₁ by blocking synthesis of new receptors without influencing basal receptor degradation, and both ConA and SR would increase surface CB₁ by inhibiting constitutive endocytosis. Cell counts were also monitored to provide a gross indication of altered cell health or proliferation rate.

To select appropriate working concentrations of these drugs, 6 h concentration response curves were produced (Figure 3.7A-D). This timepoint was relevant as changes in CB₁ expression were expected to occur over this period and it was therefore intended that drugs
would be applied for similar time spans in subsequent assays. Indeed, in this set of preparatory experiments all drugs produced the anticipated changes in CB₁ expression.

Monensin produced a maximal effect at 100 nM and 1 μM (Figure 3.7A). A trend towards reduced cell density with increasing monensin concentrations is evident, becoming significant at 1 μM ($p = 0.015$). In addition, unusual morphology, namely smaller cell size and cytoplasmic accumulation of what appeared to be numerous vesicles, was observed at 1 μM (Figure 3.7E). 100 nM was therefore selected for use in subsequent experiments.

Over the range of CHX concentrations tested, 10 and 100 mg/L both produced highly significant reductions in total CB₁ (both $p = 0.0002$) with 100 mg/L appearing to have elicited a near-maximal effect (Figure 3.7B). A small but significant decrease in cell density was observed from 100 ng/L, preceding any significant change in CB₁ expression. This suggests CHX might influence the cell proliferation rate even at low concentrations. In latter pilot experiments both 10 and 100 mg/L were tested, however much greater reductions in cell number were observed with 100 mg/L, an effect perhaps revealed because more washing steps were implemented due to the design of the particular experiments. 100 mg/L CHX also induced alterations in cell morphology including markedly smaller cell size and membrane blebbing (Figure 3.7E). This indicated cells had reduced adherence and/or viability at this concentration and CHX was used at 10 mg/L in later experiments.

ConA and SR exhibited similar concentration response profiles, both demonstrating maximal effects on surface CB₁ expression at 100 nM and 1 μM (Figure 3.7C-D). Slight but non-significant increases in cell density also occurred ($p = 0.20$ and 0.17 respectively). Both drugs were used at 1 μM in subsequent experiments although different SR concentrations were sometimes applied, as indicated.
At the drug concentrations selected for co-stimulation experiments, cells appeared to have maintained normal morphology indicating their viability had not been markedly compromised by the drug treatment (Figure 3.7E).
Figure 3.7 Selection of monensin, CHX, ConA and SR concentrations for subsequent co-stimulation experiments

(A-D) Surface rCB₁ expression levels and cell density responses to varying concentrations of monensin (A), CHX (B), ConA (C) and SR (D) for 6 h. Arrows indicate drug concentrations selected for subsequent experiments. (E) Brightfield images of cells treated with vehicle or drugs at indicated concentrations. Bars, 15 µm.
CHAPTER THREE
QUANTITATIVE ASSAY DEVELOPMENT

A B

Surface CB1 or Cell count (% Vehicle-treated)

Log [Monensin] (M)

***

Log [CHX] (g/L)

***

Surface CB1 or Cell count (% Vehicle-treated)

Log [ConA] (M)

***

Log [SR] (M)

***

E

Vehicles

Monensin 100 nM

Monensin 1 µM

CHX 10 mg/L

CHX 100 mg/L

ConA 1 µM

SR 1 µM

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**DISCUSSION**

The experiments in this chapter demonstrate the validation and optimisation of the methodologies that underpin the subsequent research presented in this thesis.

Initially, antibodies suitable for detecting HA tagged and native CB₁ were selected. The anti-HA antibody selectively and effectively detected surface and total HA-CB₁, as did two antibodies directed against the CB₁ C-terminus and one against the N-terminus that had been produced and gifted to the laboratory by a fellow researcher. However, it was disappointing to note that four commercially available antibodies directed against the CB₁ N-terminus failed to detect CB₁ and the majority exhibited non-specific staining by immunocytochemistry when applied under permeabilising conditions. Although it is possible the presence of the HA tag on CB₁ may have inhibited binding by these antibodies, further investigation with untagged endogenously expressed CB₁ using a range of techniques (immunocytochemistry, immunohistochemistry and western blotting) also found these antibodies to be ineffectual (see Grimsey et al., 2008). Interestingly, our lab had found that a previous lot of the BioSource antibody had worked as expected (Park et al., 2003; Graham et al., 2006) but the more recently purchased aliquot (used here) gave markedly different results. These findings suggest that antibody specificity and efficacy claimed by manufacturers cannot necessarily be relied upon and that thorough testing of newly acquired antibodies by researchers is advisable (whether or not used successfully in the past).

The temperature at which live antibody labelling to detect surface receptors would best be performed was next investigated. Although the greatest intensity of staining was observed at 37°C, constitutive internalisation during the 30 min incubation period was noted, as was extensive internalisation when cells were incubated with agonist (as would be anticipated based on previous studies suggesting that CB₁ rapidly internalises in response to agonist stimulation). Therefore, although live labelling at 37°C would be useful for assays in which receptors were to be tagged prior to drug treatment, this would not be appropriate when assaying for net surface
receptors present at the end of an experiment. Staining in the presence or absence of agonist was therefore also compared at RT and 4°C (both following a brief incubation on ice to rapidly cool cell media). No CB₁ trafficking was observed at either temperature, however the overall level of staining was markedly reduced in the 4°C condition. Therefore RT was selected for use in subsequent experiments. Inhibition of membrane trafficking below a certain temperature is likely due to a change in membrane dynamics such as reduced fluidity and/or the inhibition of specific proteins regulating transport (von Zastrow and Kobilka, 1994).

Having selected appropriate antibodies and staining conditions, a novel method for the quantification of receptor trafficking utilising a high-content imaging system and a high-throughput image analysis was developed and successfully applied. The method, referred to as TGVC, is based on widefield fluorescence imaging and subsequent quantification with a measure of the total intensity of signal, averaged between the number of cells in the image. As well as being in agreement with previously published data (Coutts et al., 2001; Leterrier et al., 2004), the results of an internalisation timecourse obtained by TGVC were well correlated to those for Granularity, an established assay for the measurement of internalisation (Conway et al., 1999; Lee et al., 2006). The observed differences in internalisation rate detected by the two methods is likely related to the well-documented phenomenon of receptor clustering observed during the formation of clathrin-coated pits (Goldstein et al., 1985) that may result in the detection of increased numbers of granules, despite vesicle scission from the plasma membrane not yet having occurred. Therefore the granularity method may erroneously indicate a more rapid rate of endocytosis than has actually occurred. The TGVC method developed here is a more direct measure of endocytosis as it will continue to detect cell-surface receptors up to the point at which the receptor N-terminus is no longer accessible to the extracellular milieu. As TGVC measures the total fluorescent signal per cell and is not dependent on a particular morphological change in staining, as is Granularity, this assay may be utilised to investigate a range of receptor trafficking events. This versatility is facilitated by the conditions under which immunocytochemical labelling is performed.
The TGVC method possesses a number of important advantages to some other currently implemented approaches. The relatively straightforward sample preparation and high-throughput nature of analysis utilised enables the rapid assessment of large amounts of data. In fact, the approach could be scaled up further still by using plate formats with greater numbers of wells (Yarrow et al., 2003). Importantly, unlike manual image acquisition and analysis approaches, use of an automated system eliminates the risk of unintentional selection bias. These are strong advantages over labour intensive methods such as ratiometric confocal image analysis and western blotting. ELISA-based assays, while able to be processed at high-throughput, may not provide sufficient information for certain applications, for instance changes in cell number across experimental conditions can not necessarily be controlled for. Flow cytometry generally employs similar receptor detection techniques to those applied in TGVC, however application of the method can be restricted by cell types (ie. irregularly shaped or particularly large cells) and the limited scope for automation of sample loading (Waller et al., 2004). Although granularity-based assays have already been implemented for detecting receptor trafficking at high-throughput, the assay outcome (change in cellular receptor “granules”) is not necessarily appropriate for all investigations of internalisation (for example, with no upper limit to the theoretical number of receptor granules per cell it may be difficult to determine whether an agonist has induced a maximal response), nor other aspects of receptor trafficking such as recycling or degradation. Fluorescent plate readers, while potentially very useful when high-intensity signals are to be quantified, were found not to be sensitive enough to successfully quantify fluorescent immunocytochemistry arising from cell surface receptor staining in pilot studies for this thesis (FLUOstar Optima, BMG Labtechnologies, Offenburg, Germany).

Perhaps the greatest advantage of the method developed here is its versatility for investigating a wide range of research questions. Using different combinations of immunocytochemical labelling conditions, many aspects of receptor trafficking events can be investigated, including internalisation (live antibody feeding prior-to or after drug exposure), constitutive internalisation
(live antibody pulses), recycling (live antibody feeding prior to internalisation or after drug exposure) and inverse-agonist induced cell surface upregulation (live antibody feeding after drug exposure) – all of which call for detection of primary antibody under non-permeabilising conditions – as well as downregulation, which calls for detection of total cellular receptor. Further, the technique could be easily adapted to most other image acquisition platforms and analysis software (eg. ImageJ, http://rsbweb.nih.gov/ij/). So long as a reliable antibody to the protein of interest is available, and a signal detectable above background, this method is applicable in a wide range of model systems, including cultured cells expressing endogenous receptor and intact tissue. The antibodies used in this study produced very low levels of non-specific staining that were near to the detection limit of the Discovery-1™ microscope and quantified to be marginally greater than 0% of positive control staining. The thresholding and background subtraction method utilised in the TGVC calculation therefore successfully approximated true zero, however it is important to note that antibodies exhibiting lesser specificity may produce a measurable signal with the TGVC method and this should be taken into account during analysis so as not to artefactually reduce the measurement window.

The TGVC method is therefore a novel, high-throughput, versatile approach to intracellular receptor trafficking quantification that meets the criteria set out in aim one of this thesis. Namely it facilitates accurate and sensitive quantification of receptors, demonstrated in the reproducibility of data between experimental repeats and quantification approaches (ie. versus granularity), and does so at high-throughput and with little opportunity for introduced human bias. Importantly, the TGVC method is logically appropriate and the quantification achieved reflects the trends observable by eye with immunocytochemistry. Although only internalisation was demonstrated in the sub-section of this chapter specifically dedicated to optimisation of this method, the ability to quantify total and intracellular CB1 was achieved subsequently when developing a proteolysis approach for selectively labelling intracellular receptors.
Thus, the newly developed quantification method was next utilised to optimise a method for the selective detection of intracellular receptors. Utilising proteolytic enzyme trypsin, the extracellular tail of HA-CB₁ was cleaved such that it was no longer recognisable to the HA antibody used. Although not verified here, this technique was found in later experiments to also render native surface CB₁ unrecognisable to the Mackie-NT CB₁ antibody (pg. 98). It is anticipated that this method would also be applicable to receptors other than CB₁ so long as the receptor of interest had an accessible trypsin cleavage residue that would disrupt the associated antibody recognition site. It cannot be determined from the experiments performed here exactly how distal from the plasma membrane the trypsin cleavage site must be for efficient digestion under the conditions optimised here, and indeed CB₁ is known to have a relatively long N-terminus in comparison to many other receptors (Wallin and von Heijne, 1995). These experiments also do not reveal the extent to which the receptor extracellular loops or their associated post-translational modifications may be altered. Depending on whether these regions are affected, in future studies it may be interesting to investigate the effect of receptor extracellular terminus trypsinisation on signalling and other receptor properties. Indeed, it has already been established by a mutagenic approach that the CB₁ N-terminus is not required for receptor signalling (Andersson et al., 2003). In this study, however, this method will be used solely to immunocytochemically isolate cytoplasmic receptors from those situated at the plasma membrane.

Finally in this chapter, appropriate concentrations of pharmacological reagents likely to be of use in later experiments were selected. Surface or total CB₁ was monitored in the presence of a range of drug concentrations in comparison to vehicle for 6 h. The concentrations selected for continued use gave maximal, or near-maximal CB₁ responses with little or no change in cell density and no visible changes in cell morphology. The latter two observations indicated that cells were healthy and had not undergone marked differentiation, reducing the likelihood that the drugs might have unintended effects on the trafficking pathways of interest. The
concentrations selected were similar to or less than those used in previous receptor trafficking studies (see Table 3.2).

At the foundation of any scientific endeavour are the methods and techniques employed to convert the phenomenon of interest to interpretable outputs. As demonstrated in this chapter, a significant degree of care was taken to select and optimise the methods intended for use in this study. These techniques act as the basis for the detailed investigations into CB₁ trafficking presented in chapters four and five.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Selected concentration</th>
<th>Previously used concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monensin</td>
<td>100 nM</td>
<td>500 nM (Ko et al., 1999), 70 nM (Leterrier et al., 2004), 50 µM (Veyrat-Durebex 05)</td>
</tr>
<tr>
<td>CHX</td>
<td>10 mg/L (35.5 µM)</td>
<td>70 µM (Hsieh et al., 1999), 70 µM (Leterrier et al., 2004), 40 µM (Tappe-Theodor et al., 2007)</td>
</tr>
<tr>
<td>ConA</td>
<td>1 µM (26.5 mg/L)</td>
<td>250 mg/L (Tang et al., 2000), 25 mg/L (Baig et al., 2002)</td>
</tr>
<tr>
<td>SR</td>
<td>1 µM *</td>
<td>100 nM (Hsieh et al., 1999), 1 µM (Coutts et al., 2001), 10 µM (McDonald et al., 2007)</td>
</tr>
</tbody>
</table>

Table 3.2 Drug concentrations selected for use in this study in comparison with previously published studies

* Also used at lower concentrations in some experiments, as indicated.
CHAPTER FOUR

INVESTIGATIONS INTO CB₁ TRAFFICKING AND THE ROLE OF THE INTRACELLULAR POOL

INTRODUCTION

As a highly prevalent receptor that modulates a range of brain and systemic functions, CB₁ is currently of significant interest as a pharmaceutical target. Cellular control of receptor intracellular trafficking plays a central role in receptor function, particularly with regard to the capacity for resensitisation following agonist exposure. It is becoming increasingly apparent that perturbation of these pathways may contribute to disease processes (reviewed in Conn et al., 2007).

While the study of CB₁ intracellular trafficking is in its infancy in comparison to many other receptors, the subject is an area of intense interest and has been the focus of a number of research reports in recent years. CB₁ is known to be expressed at the surface of cells and undergo constitutive and agonist-induced endocytosis (Coutts et al., 2001; Hsieh et al., 1999; Leterrier et al., 2004). Downregulation of CB₁ following chronic agonist stimulation has also been widely reported (reviewed in Sim-Selley, 2003), and recently GASP-1 (Martini et al., 2007; Tappe-Theodor et al., 2007) and AP3 (Rozenfeld and Devi, 2008), adaptor proteins associated with sorting and delivery of receptors to lysosomes, were demonstrated to interact with CB₁.

However, in the brain (eg. Pettit et al., 1998; Tsou et al., 1998; Katona et al., 1999), as well as in endogenously expressing (McIntosh et al., 1998; Graham et al., 2006) and transfected
(Rinaldi-Carmona et al., 1998; Hsieh et al., 1999; Leterrier et al., 2004; Tappe-Theodor et al., 2007; Wu et al., 2008) immortalized cell lines, a significant proportion of CB₁ is located in an “intracellular pool” in the cytoplasm. This intracellular pool displays only minimal colocalisation with protein synthesis-associated organelles (Leterrier et al., 2004; Rozenfeld and Devi, 2008). This observation, combined with the ability of the receptor to constitutively endocytose (Leterrier et al., 2004) and correlation of results with other receptors that exhibit similar phenotypes (eg. Parent et al., 2001; Miserey-Lenkei et al., 2002; Marion et al., 2004) have led to the inference that this intracellular pool serves as a reservoir of endocytic origin. This reservoir may function as a source from which surface CB₁ is replenished to replace internalised receptor (Leterrier et al., 2004), suggesting that CB₁ exhibits a recycling phenotype. A handful of other studies also provide evidence towards recycling following agonist-induced internalisation (Hsieh et al., 1999; Martini et al., 2007).

In contrast to this recycling hypothesis, pilot experiments towards this project indicated that CB₁ did not recycle, revealing the need for a thorough and integrated study of CB₁ intracellular trafficking. The research described in this chapter therefore provides a detailed characterization of CB₁ intracellular trafficking and investigates the role of the intracellular pool in four cell lines, one of which expresses CB₁ endogenously.

**METHODS**

A detailed account of materials and methods employed are provided in chapters two and three.

The majority of the experiments in this chapter were performed with the rCB₁ HEK cell line. To ensure that the results were not cell-type or species specific, experiments central to the findings were also performed on the pplss-hCB₁ HEK, hCB₁ CHO, hCB₁ AtT-20 and Neuro-2a cell lines. Unless otherwise noted, ‘HEK’ refers to the rCB₁ cell line, whereas ‘HEK pplss’ refers to the pplss-hCB₁ line.
Drug treatments are described in the text. To quantify or visualise intracellular receptor, cells were briefly exposed to trypsin in order to cleave surface antibody recognition sites (for method optimisation see pg. 64). Unless indicated, anti-HA or anti-CB₁ Mackie-NT (Neuro-2a experiments only) primary antibodies were incubated with live cells at RT following drug stimulations to detect surface CB₁. Some assays utilised antibody “feeding” whereby surface CB₁ was labelled with primary antibody at 37°C prior-to or during drug treatment. Secondary antibody was subsequently applied to live cells at RT to detect primary antibody-labelled CB₁ at the cell surface, or following fixation under permeabilising conditions to detect both surface and internalised primary antibody-labelled CB₁. To detect total CB₁, anti-HA or anti-CB₁ L15 (Neuro-2a experiments only) primary antibodies were incubated with fixed cells under permeabilising conditions, after which secondary antibody was applied.

Following image acquisition with Discovery-1™, assays were quantified in MetaMorph® with the TGVC method as described in chapter three (see pg. 44 onwards). Western blotting and confocal microscopy were also utilised.

**Results**

**CB₁ is present both at the cell surface and in a large intracellular pool in transfected and endogenously expressing cell lines**

The subcellular localisation of CB₁ in each of the model systems was investigated first. Consistent with previous reports (eg. Leterrier et al., 2004), CB₁ 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 (Figure 4.1 A). In order to quantify the proportion of CB₁ in the cytoplasm versus at the plasma membrane, CB₁ staining was assessed with and without a brief exposure to trypsin. CB₁ detected in trypsin-treated cells was divided by total CB₁ to give the proportion of intracellular CB₁ and was thereby determined to account for 25-70% of total expression, depending upon the cell type tested.
(Figure 4.1Bi). The variations in the proportion of intracellular CB₁ were not related in any obvious way to total expression levels in the cell lines tested (Figure 4.1Bii).

When lysates from the rCB₁, HEK, hCB₁, AtT-20, and hCB₁ CHO cell lines were analyzed by western blot, a prominent species of approximately 64 kDa was detected (Figure 4.1 C). Two smaller bands of approximately 50 and 45 kDa were also present. These were of low abundance in the HEK and CHO cells but were more prominent in the AtT-20 cell lysates. 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 CB₁ 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 F 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 rCB₁, 53 kDa). This suggests, at least in the HEK and CHO cell lines, that the majority of intracellular pool CB₁ is full-length and similarly glycosylated to surface CB₁. A lack of detectable signal from HEK cells not expressing HA-CB₁ confirmed the specificity of the anti-HA antibody in western blotting (Figure 4.1 Civ). Unfortunately, none of the CB₁ antibodies tested proved suitable for probing western blots, so the Neuro-2a cell line was not investigated with this method.
Figure 4.1 CB₁ localisation in HEK, CHO, AtT-20 and Neuro-2a cells

(A) Confocal micrographs of CB₁ in five cell lines. Live cells were incubated with HA primary antibody and, following fixation, Alexa Fluor® 488-conjugated secondary antibody to detect surface HA-CB₁ (green). Cells were then fixed for a second time and incubated again with HA primary then Alexa Fluor® 594-conjugated secondary antibodies to detect total/intracellular HA-CB₁ (red). Bar, 10 μm. (B) Intracellular CB₁ as a percentage of total CB₁ expression in the same cell type (i) and total CB₁ as a percentage of HEK rCB₁ total expression (ii). (C) Western blots for HA-CB₁ in lysates from HEK (i), AtT-20 (ii), and CHO (iii) cells treated with trypsin and/or PNGase. Lanes are from the same film exposure but have been re-arranged for presentation. No signal was detected when un-transfected HEK cell lysate was probed (iv, “WT”). Protein standard marker indicated in kDa.
CHAPTER FOUR

CB, TRAFFICKING AND ROLE OF INTRACELLULAR POOL

A

HEK

HEK pplss

AtT-20

CHO

Neuro-2a

B

100

75

50

25

0

Intracellular CB1 (% Total)

HEK  HEK pplss  AtT-20  CHO  Neuro-2a

1300

100

75

50

25

0

Total CB1 (% HEK)

HEK  HEK pplss  AtT-20  CHO  Neuro-2a

C

PNGase:

-  -  +  +

-  +  -  +

-  -  +  +

-  +  -  +  WT CB1

Trypsin:

-  +  -  +

-  +  -  +

-  -  +  +

-  +  -  +

84
CB₁ undergoes rapid agonist-induced internalisation

In agreement with preceding reports (eg. Rinaldi-Carmona et al., 1998; Hsieh et al., 1999) and as already mentioned in chapter three (pg. 60), rapid internalisation of CB₁ following the application of agonist was observed. This endocytosis from the plasma membrane was dependent on the concentration of agonist applied and the duration of stimulation (Figure 4.2A-C). The logEC₅₀s for HU and WIN internalisation at 60 min were -10.0 ± 0.099 M and -7.8 ± 0.092 M respectively, indicating that HU induced internalisation approximately 100-fold more potently at this timepoint. WIN was observed to induce a greater maximum rate of internalisation (one-phase exponential decay; t½: HU 5.16 ± 0.76 min; WIN 2.84 ± 0.57 min; \( p = 0.044 \)) and trended towards a greater efficacy at the highest ligand concentrations tested. The logEC₅₀s for internalisation closely correlated with those for inhibition of forskolin-stimulated cAMP levels (Figure 4.2D; HU -9.93 ± 0.24 M; WIN -7.94 ± 0.19 M).
Figure 4.2 HU and WIN-induced CB₁ Internalisation

(A-B) Timecourse of rCB₁ internalisation in HEK cells with HU (A) or WIN (B) at indicated concentrations. (C) Internalisation concentration responses for HU and WIN with 60 min agonist stimulation. (D) Concentration responses for inhibition of forskolin-stimulated cAMP levels by 15 min HU and WIN stimulation (note: cAMP assays were carried out by Dr Debbie Hay).
Surface repopulation of CB₁ following agonist stimulation is dependent on ratio of agonist to inverse-agonist

Having characterised agonist-induced internalisation, the recovery of surface receptors following internalisation with HU and WIN was investigated. After 15 min of 10 nM HU stimulation (to induce near-maximal internalisation), two washes in experimental media at 37°C and replacement with media containing only vehicle, no repopulation of the cell surface was detected over the next five hours (Figure 4.3A). However, if the replacement media contained 100 nM SR (a CB₁ inverse-agonist), slow surface repopulation was observed (linear regression, 12.0 ± 1.4%h⁻¹). Despite an equivalent extent of internalisation, the degree of repopulation following SR treatment was inhibited at a higher agonist concentration (100 nM; Figure 4.3A). As shown in Figure 4.3B, the extent of receptor repopulation following internalisation with either HU or WIN exhibited a strong dependence on the concentration of SR applied. Interestingly, even though HU and WIN were applied at approximately equipotent concentrations, SR induced surface repopulation with greater potency following internalisation with WIN than HU.

This data suggested that several washes with media were insufficient to completely remove agonist, and that in the absence of a sufficient concentration of SR to compete for binding sites, receptors reaching the cell membrane were rapidly internalised by residual agonist. Consistent with this hypothesis, the surface was also replenished with CB₁ if incubated with 1 μM ConA (an inhibitor of internalisation) following agonist-induced internalisation (Figure 4.3A).

To confirm this hypothesis, the process was visualized by exposing the living cells to primary antibody following agonist stimulation and internalisation (Figure 4.3C). Immediately after 15 min 10 nM HU stimulation very little receptor was detected, which is consistent with near-complete CB₁ internalisation (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 CB₁, which is consistent with receptors reaching the cell membrane and binding primary antibody, but subsequently internalising (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 repopulating CB₁ was held at the surface. However, a significant proportion was now internalised and appeared in intracellular vesicles (iv).
Figure 4.3 CB₁ cell surface repopulation following agonist-stimulated internalisation

(A and B) Surface rCB₁ in HEK cells following 15 min agonist stimulation, washing, and incubation with SR 100 nM, ConA 1 µM, or vehicle for 0-5 h (A) or SR at varying concentrations for 2 h (B).

(C) Widefield images of rCB₁ 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 CB₁ immediately following agonist stimulation. In this experiment, only CB₁ delivered to the cell surface subsequent to agonist-induced internalisation is labelled with primary antibody. After fixation, secondary antibody was applied under permeabilising conditions. Bar, 15 µm.
CHAPTER FOUR
CB, TRAFFICKING AND ROLE OF INTRACELLULAR POOL

A

B

C

i  HU 10 nM then Vehicle (4°C)

ii  HU 10 nM then Vehicle

iii  HU 10 nM then SR 1000 nM

iv  HU 100 nM then SR 1000 nM

Surface CB1 (% Basal)

HU 10 nM then SR 100 nM
HU 100 nM then SR 100 nM
HU 10 nM then Vehicle
HU 100 nM then ConA 1 μM

HU 100 nM then SR 2 h
WIN 1 μM then SR 2 h

Surface CB1 (% Vehicle)

-10
-9
-8
-7
-6
Log [SR]
Antibody live-feeding indicates internalised CB₁ does not recycle and is instead degraded

While the assays published previously and thus far described in this chapter measured net surface repopulation, the results of which have been taken to indicate that CB₁ recycles (eg. Hsieh et al., 1999), 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 internalisation. A previously established “antibody feeding” technique (Cao et al., 1999) was adapted to investigate whether antibody-tagged CB₁ returned to the surface following agonist-induced internalisation. This technique labels receptors with primary antibody prior to agonist exposure, and it was established in chapter three that this does not alter the rate of internalisation (pg. 61).

Under conditions where repopulation of the surface with CB₁ was observed (internalisation with 10 nM HU, followed by incubation with 1 μM SR), no recovery of antibody-tagged CB₁ to the surface was detected for up to six hours (Figure 4.4A, “Recycling”). Even in the presence of inverse-agonist, antibody-tagged CB₁ continued to internalise, suggesting that CB₁ 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 repopulation, cells were again exposed to antibody at the end of the experiment and repopulation was indeed observed (Figure 4.4A, “Repopulation a”). The CB₁ surface repopulation observed without the antibody live-feeding step at the start of the experiment was not significantly different (Figure 4.4A, “Repopulation b”, p = 0.85). Analogous results were obtained when CB₁ was internalised with 1 nM HU or 100 nM WIN (data not shown).

To ensure this method could detect receptor recycling, this assay was repeated in HEK cells expressing HA-tagged human D₁, a receptor that has previously been reported to recycle (Vickery and von Zastrow, 1999). As shown in Figure 4.4B, following antibody feeding, 10 μM
dopamine-induced internalisation, and washout with D₁ antagonist (SCH23390 10 μM), recycled antibody-tagged D₁ receptors were clearly detected at the cell surface. It was also notable that the rate of D₁ surface repopulation was much more rapid than for CB₁ with the cell surface being essentially repopulated in less than an hour (one-phase exponential association from t₀, t½ = 0.20 ± 0.025 h, 2.9 ± 0.30 h respectively; p = <0.001).

When antibody-bound receptor was detected with secondary antibody under permeabilising conditions to label both surface and internalised receptors, total immunoreactivity associated with D₁ was unchanged over the timecourse studied (p = 0.96). In contrast, that associated with CB₁ progressively decreased (plateau then one-phase exponential decay, X₀ = 0.17 ± 0.076 h, t½ = 1.04 ± 0.38 h) until it was equal-to or less than the limit of detection, suggesting that internalised CB₁ had degraded (Figure 4.4C). The localisation of internalised receptor was also different between the two receptors, with CB₁ internalised to endosomes of varying size and staining intensity scattered diffusely in the cytoplasm, whereas internalised D₁ endosomes clustered in a well-defined perinuclear area (Figure 4.4D), suggesting that CB₁ enters a different post-endocytic trafficking pathway to D₁.

To ensure that the failure of CB₁ to recycle was not unique to HEK cells, live antibody-feeding experiments were also carried out on Neuro-2a cells and the transfected CHO and AtT-20 cells. In all cell lines the cell surface was repopulated with CB₁ after 10 nM HU-induced internalisation and washout with 1 μM SR (Figure 4.4E “Repopulation”), however no return of antibody-tagged CB₁ to the plasma membrane was detected (Figure 4.4E “Recycling”).
Figure 4.4 Antibody live-feeding indicates endocytosed CB₁ does not recycle and is instead degraded

(A and B) Surface rCB₁ (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”), at the end of all drug treatment (“Repopulation b”), or both prior to and at the end of drug treatment (“Repopulation a”). Secondary antibody was applied under non-permeabilising conditions to detect surface receptor. (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 permeabilising conditions to detect remaining primary antibody in the entire cell. (D) Bars, 15 μm. (E) Surface CB₁ in AtT-20, CHO and Neuro-2a cells treated as in A with 5 h SR incubation.
CHAPTER FOUR

**CB, TRAFFICKING AND ROLE OF INTRACELLULAR POOL**

![Graphs and images showing trafficking and role of intracellular pool](image)

**A**
Surface CB1 (% Basal) 15 min Agonist Inverse-Agonist (h)

**B**
Surface D1 (% Basal) 15 min Agonist Antagonist (h)

**C**
Total Live-fed Antibody (% Basal) 15 min Agonist Inverse-Agonist or Antagonist (h)

**D**
Basal Agonist 15 min Inverse-agonist 6 h
Basal Agonist 15 min Antagonist 2 h
Basal

**E**
Surface CB1 (% Basal) for AtT-20, CHO, Neuro-2a
Constitutively internalised CB₁ is degraded following endocytosis and does not accumulate to form the intracellular pool

CB₁ is generally considered to be a constitutively active receptor (reviewed in Pertwee, 2005) and ligand-independent constitutive internalisation is associated with this property (Leterrier et al., 2004). As inferences from previous studies have suggested that the CB₁ intracellular pool is formed as a result of constitutive internalisation and contributes to a constitutive recycling pathway, these pathways were examined directly. rCB₁ 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-permeabilising conditions to detect only primary antibody-bound receptor remaining on the surface, CB₁ underwent constitutive endocytosis with a half-life of 1.29 ± 0.12 h (one-phase exponential decay; Figure 4.5A). 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 CB₁ population had turned-over. In order to determine the fate of the endocytosed receptors, the secondary antibody was applied under permeabilising conditions and a progressive reduction in signal was noted (plateau then one-phase exponential decay, X₀ = 0.33 ± 0.29 h, t½ = 1.44 ± 0.17 h) indicating that CB₁ was likely degraded following constitutive internalisation, rather than recycled to the cell surface or sequestered in the intracellular pool.

To investigate whether the constitutively internalised receptor recycled, primary antibody was applied to live cells for an extended period (4 h), and the tagged receptors still at the cell surface at the end of this time were allowed to internalise (incubation in vehicle, 2 h) before SR 1 μM was applied for 6 h and the antibody-tagged CB₁ was monitored for any return to the cell surface and the longevity of signal in the cytoplasm. Again, no recovery of antibody-tagged CB₁ to the plasma membrane was observed, and instead both the surface and intracellular signal was progressively lost during the vehicle and SR incubation periods (Figure 4.5B).
As it was recognised that it might be difficult to detect a low level of recycling with the previous paradigms, in which signal from recycled receptor would need to be detected over and above that remaining at the surface following internalisation, a modified version of a previously applied surface antibody “stripping” protocol was employed (Vargas and Von Zastrow, 2004; Martini et al., 2007). In this experiment cells were incubated with primary antibody for 30 min at 37°C and allowed to constitutively internalise for 1 h. At the conclusion of this internalisation, cells were exposed to trypsin to cleave any antibody bound to remaining surface receptors and subsequently incubated with inverse-agonist for 6 h to trap any recycling receptors at the cell surface. When performed with HEK pPliS-hCB1 cells, no recycling was detected in the quantification (Figure 4.5C). However close inspection of the images, when displayed at much higher brightness levels than appropriate for basal receptor expression, revealed an extremely small extent of recycling (Figure 4.5D). This was estimated by eye to represent less than 1% of starting surface expression. This experiment was repeated with agonist-induced internalisation and the rCB1 HEK cell line, but no evidence of recycling was detected either in the quantification or by visual inspection (data not shown).
Figure 4.5 CB₁ undergoes constitutive endocytosis but is subsequently degraded

(A and B) Surface rCB₁ in HEK cells was labelled with primary antibody and allowed to internalise for 0-8 h (A), or 2 h then exposed to inverse-agonist for 0-6 h to determine whether constitutively internalised CB₁ recycled (B). Secondary antibody was then applied under non-permeabilising (“Surface”) or permeabilising (“Total”) conditions. (C and D) Surface pplss-hCB₁ in HEK cells was labelled with primary antibody and allowed to internalise for 1 h. Cells were exposed to trypsin (“Strip”) or EDTA only (“No Strip”) and subsequently incubated with inverse-agonist for 6 h to determine whether constitutively internalised CB₁ recycled. (D) Images are provided at standard (i) and high (ii) brightness levels. Bar, 10 µm.
Blockade of constitutive internalisation results in an upregulation of surface and total CB₁, which is prevented by protein synthesis inhibition

The interaction of inverse-agonist with CB₁ stabilizes the receptor in an inactive conformation and prevents constitutive signalling and constitutive endocytosis (Pertwee, 2005), resulting in the accumulation of receptors at the plasma membrane. Consistent with this model, surface CB₁ is upregulated when cells are incubated with 1 μM SR (one-phase exponential association, \( t_{1/2} = 2.74 \pm 0.33 \) h, Figure 4.6A). Under these conditions, total cellular CB₁ was also increased (\( t_{1/2} = 2.82 \pm 0.40 \) h), indicating that at least a proportion of the receptors stabilized at the surface derive from the synthetic pathway. A highly similar rate of SR-induced upregulation was observed when cells were incubated with 1 μM ConA (\( p = 0.54 \)), while treatment with monensin (which inhibits vesicle acidification and thereby trafficking to the cell surface, Mollenhauer et al., 1990) inhibited surface upregulation (Figure 4.6C). These effects were also demonstrated with concentration responses in chapter three (pg. 68-72). As well as changes in surface and total receptors, the size of the intracellular pool was monitored directly by performing permeabilising immunocytochemistry on cells briefly treated with trypsin (as described above). No significant change in the size of the intracellular pool following incubation for 5 h with SR (\( p = 0.66 \)) was observed, despite there being significant increases in both cell surface and total receptor expression (both \( p = 0.0005 \); Figure 4.6B, left panel). Similar results were obtained with endogenous CB₁ in Neuro-2a cells (Figure 4.6B, right panel).

The lack of change in intracellular pool size suggests that surface CB₁ upregulation 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, cycloheximide (CHX). CHX (10 mg/L) completely inhibited surface and total CB₁ upregulation following SR treatment (Figure 4.6D). This finding was replicated in the three other cell models at a single time point (Figure 4.6E). As shown in chapter three, cells appeared morphologically normal and cell density was not markedly reduced after 6 h at this CHX concentration (pg. 69).
This experiment was repeated by western blot with HEK cells, comparing total CB₁ with that still detectable in trypsin-treated cells (ie. intracellular pool only) and again no change in the size of the intracellular pool with SR treatment was observed. However, the intracellular pool-associated signal decreased with CHX treatment regardless of the presence of SR (Figure 4.6F). Interestingly, the ~45 kDa band was eliminated following CHX treatment.

CHX also completely prevented the repopulation of surface CB₁ following agonist-induced internalisation in HEK cells (data not shown).
Figure 4.6  Inverse-agonist induced cell surface upregulation is blocked by protein synthesis inhibition

(A) Surface and total rCB₁ expression in HEK cells following 0-18 h incubation with 1 µM SR. (B) Surface, intracellular, and total CB₁ expression in HEK and Neuro-2a cells following incubation with vehicle or SR for 5 h. Data is normalized to total CB₁ expression in vehicle-treated cells. (C) Surface CB₁ following 5 h incubation with SR and/or monensin. (D) Surface (i) or Total (ii) CB₁ in HEK cells following 0-5 h incubation with SR and/or CHX. (E) Surface CB₁ in AtT-20, CHO and Neuro-2a cells following 5 h incubation with SR and/or CHX. (F) Western blot of CB₁ in lysates from HEK cells treated with SR and/or CHX for 6 h. Samples not exposed to trypsin demonstrate total CB₁, while those treated with trypsin reveal intracellular CB₁. Films were exposed for standard (upper panel) or extended duration (lower panel). Protein standard marker indicated, kDa.
Surface, but not intracellular CB₁, is degraded with chronic agonist stimulation

A common consequence of chronic GPCR stimulation is overall receptor downregulation. As demonstrated in Figure 4.7A-B, this is indeed the case for CB₁, and the extent and timecourse of downregulation are dependent on the concentration of agonist applied. The maximum half-life of degradation observed was 0.82 ± 0.29 h with 1 µM HU (one-phase exponential decay; Figure 4.7A). Interestingly, the chronic agonist effect reached a plateau at 55.5 ± 2.5% of the starting expression level. Similar results were observed with WIN (Figure 4.7B) and with Neuro-2a cells (Figure 4.7C).

Incubating with agonist for longer time periods (up to 48 h) or by replenishing the incubation media with fresh agonist trended towards producing slightly more degradation. However, the extents were not significantly different and more than 45% of the starting CB₁ expression level remained (Figure 4.7D, \( p = 0.062 \)). The question of whether SR-induced surface receptor upregulation prior to the addition of chronic agonist would alter the extent of degradation was also investigated. However the amount of receptor remaining following chronic agonist treatment did not change (Figure 4.7E, \( p = 0.088 \)), suggesting that the influx of receptors to the cytoplasm upon agonist stimulation was not saturating the degradation pathway. This was confirmed with an antibody-feeding experiment, whereby the fate of surface receptors labelled with primary antibody prior to agonist stimulation was observed. Indeed, the antibody-receptor signal was lost well within the 6 h time period studied (Figure 4.7F). By qualitative observation all cells appeared to respond uniformly (Figure 4.7G), confirming that the inability to produce complete degradation was not a result of only a sub-population of cells responding to the agonist stimulation.
Figure 4.7 Chronic agonist stimulation results in CB₁ degradation

(A and B) Total rCB₁ in HEK cells treated for 0-6 h with HU at 3 concentrations (A) or for 6 h with varying concentrations of HU or WIN (B). (C) Total CB₁ in Neuro-2a cells treated for 0-6 h with 1 μM HU. Endogenously expressed CB₁ was detected with L15 primary antibody. (D) Total rCB₁ in HEK cells treated for 6-48 h with 1 μM HU. 24+24 indicates cells were treated for a total of 48 h with media replenished after 24 h. (E) Total rCB₁ in HEK cells pre-incubated with vehicle or inverse-agonist (“SR”) for 18 h, then stimulated with 1 μM HU for 6 h. (F) Experiment performed as in (E), except at the conclusion of the 18 h pre-incubation surface CB₁ was labelled with primary antibody. Secondary antibody was applied under permeabilising conditions to quantify the fate of internalised antibody-bound receptor. (G) Widefield images of total rCB₁ in HEK cells following treatment for 0 or 6 h with 1 μM HU. Bar, 10 μm.
CHAPTER FOUR
CB, TRAFFICKING AND ROLE OF INTRACELLULAR POOL

A B C

D E F

G
Intracellular CB₁ does not colocalise with Gα subunits

The experiments described herein demonstrate that the localisation of intracellular CB₁ is not influenced by agonist or inverse-agonist stimulation. However, a recent study indicated that intracellular CB₁ may be responsible for agonist-mediated signalling responses (Rozenfeld and Devi, 2008). It was therefore of interest to determine whether Gα subtypes known to mediate CB₁ signalling (Mukhopadhyay and Howlett, 2001) colocalised with the CB₁ intracellular pool.

HEK cells probed with antibodies to Gα i1, or i3 and o, demonstrated predominantly surface staining (red, Figure 4.8), and Gα labelling colocalised with surface CB₁ (detected by incubating primary antibody with live cells prior to fixation; green). Although a small amount of cytoplasmic staining was noted, no colocalisation of any of these G-protein subunits with the CB₁ intracellular pool was observed (detected by incubating primary antibody under permeabilising conditions on trypsin-treated cells; green).
Figure 4.8 Intracellular CB₁ is not colocalised with inhibitory Gα subunits

Confocal micrographs of rCB₁ HEK cells stained for surface or intracellular CB₁ (green) and Gα i1 or i3 and o (red). Bars, 10 μm.
DISCUSSION

This study has utilized both cells expressing transfected and endogenous CB₁ to investigate various aspects of CB₁ intracellular trafficking. In the cell lines studied, approximately 48% (± 20%) of CB₁-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. Proteolytic cleavage of the CB₁ extracellular domain was utilised to examine the intracellular receptor by western blotting. This approach revealed that the predominant CB₁-associated bands for total and intracellular CB₁ 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 CB₁ to the cell surface, as has been noted to occur for other receptors (eg. Compton et al., 2002). Two smaller CB₁-associated bands of low abundance were also noted whose detection was not affected by trypsin treatment, indicating that these species were likely to reside intracellularly. The ~50 kDa band appeared to represent un-glycosylated full-length receptor, whereas the ~45 kDa species appeared to be a non-glycosylated truncated form. The preferential metabolism of the 45 kDa band with CHX treatment suggested that it was unlikely this species represented full-length mature CB₁ undergoing degradation. There is some precedent for the presence of truncated CB₁ species – an N-terminally truncated variant, apparently resulting from proteolytic cleavage in the cytoplasm, has been detected in BHK cells (Nordström and Andersson, 2006). CB₁ splice variants have also been reported (Shire et al., 1995; Ryberg et al., 2005), however it is unlikely this type of mechanism is involved here because the cDNA constructs transfected into these cell lines did not contain endogenous non-coding regions. Interestingly, the 50 kDa band was not reduced with CHX treatment, and instead appeared to subtly increase in abundance. This may indicate that the application of CHX interfered with the processing and maturation of CB₁ through the ER and/or Golgi as well as protein translation.
In agreement with previous reports, CB₁ underwent rapid internalisation in response to acute agonist stimulation in a concentration-dependent manner. Agonist potencies for internalisation and inhibition of cAMP were well correlated, with HU exhibiting approximately 100-fold greater potency than WIN in both assays. Interestingly, WIN appeared to induce slightly greater rates and extents of internalisation at the highest agonist concentrations tested which may have been a result of differences in the exact CB₁ conformational states stabilised, residency time of the ligand-receptor complex and/or the downstream signalling pathways activated (Lauckner et al., 2005; McIntosh et al., 2007).

To investigate post-endocytic trafficking, internalisation was stimulated by applying agonist and the subsequent recovery of surface CB₁ was monitored. A 15 min timepoint was selected for the initial agonist stimulation as this induced a near-maximal internalisation extent at a range of agonist concentrations, however was not so long as to risk diverting potentially recycling receptors to a degradative pathway as previously reported (Hsieh et al., 1999). The plasma membrane could indeed be repopulated with CB₁, however the extent was crucially dependent on the concentration of inverse-agonist applied and less repopulation was observed as the agonist concentration was increased. Although it might be anticipated that the application of higher concentrations of agonist could re-route CB₁ from a recycling to a degradative pathway (as suggested by Martini et al., 2007), the present results demonstrate that while keeping the agonist concentration constant, surface repopulation occurred so long as the concentration of SR applied was sufficiently high to compete off agonist. This was confirmed in live antibody feeding experiments which indicated that CB₁ was constitutively delivered to the cell surface but was rapidly re-internalised unless sufficient inverse-agonist was present. This phenomenon cannot be explained by SR’s potential for interaction with intracellular receptors, as cell surface repopulation also occurred when ConA was applied (which prevents receptor internalisation), suggesting that a net accumulation of CB₁ at the cell surface was facilitated simply by the prevention of further internalisation. It was also noted that when the agonists, HU and WIN, were applied at near-equipotent concentrations (as determined by the internalisation and cAMP
concentration-response experiments), SR appeared nearly 10-fold more potent in promoting surface repopulation when applied following WIN than HU. This is perhaps suggestive of a more rapid dissociation rate for WIN. Importantly, these results indicate that simply exchanging culture media is not sufficient to washout cannabinoid agonists HU and WIN, and that subsequent agonist-induced internalisation must be prevented in order for CB1 to accumulate at the 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 lives in vivo (eg. Δ9-THC) and can take several weeks to be completely eliminated (reviewed in Ashton, 2001). 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 Raimund, 2005).

In order to investigate bona fide receptor recycling, that is, the return of the same receptor units to the cell surface following internalisation, an antibody-feeding technique which has been utilised to demonstrate receptor recycling previously was applied (Cao et al., 1999). While robust recycling of the D1 receptor was observed under these conditions, recycling of antibody-tagged CB1 was not detected in any of the cell lines used in this project. This was not due to the presence of the primary antibody inhibiting recycling, as surface repopulation was not influenced by the live antibody-feeding performed at the start of the experiment. This finding is in contrast to that of Martini et al. (2007) who observed recovery of M1 anti-FLAG antibody-tagged CB1 to the surface following stimulation with WIN in HEK cells. However, their model
system utilized an un-named "N-terminal signal sequence" which may have influenced the trafficking of their receptors. Signal sequences typically result in excessive receptor over-expression (eg. Andersson et al., 2003) which may have lead to redirection of some receptors to abnormal pathways. Unfortunately the Martini et al. (2007) report did not disclose the receptor expression levels in their cell line, nor provide a comparative image of starting surface expression levels. Indeed, in the present study when gross over-expression of CB1 was induced by the inclusion of a pplss in the receptor construct, an extremely low level of possible recycling was detected.

It is important to note that D1 recycling was much faster than the CB1 surface repopulation and internalised D1 and CB1 displayed notably different distributions, with internalised D1 congregating in a localised area of the cytoplasm but CB1 being dispersed throughout the cytoplasm. Both of these observations are consistent CB1 and D1 trafficking via different pathways. Furthermore, while the total immunoreactivity associated with internalised D1 was unchanged over the timecourse studied, that associated with internalised CB1 was lost, suggesting that CB1 was degraded. If CB1 traverses a different recycling pathway to D1 there might be opportunity for the antibody to be cleaved or dissociate from CB1, 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 CB1 (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 (Gaborik and Hunyady, 2004), with the return of surface expression to basal levels typically occurring within 60 min (eg. Seachrist et al., 2000) and 3 h (eg. Trapaidze et al., 2000; Volpicelli et al., 2002) respectively. Near-complete recovery of surface thrombin receptors resulting from intracellular pool mobilization has also been observed within 60 min (Hein et al., 1994). While the rate of CB1 surface repopulation is similar to that of recycling via the “slow” route, this pathway is independent of protein synthesis (Trapaidze et al., 2000), whereas the present results demonstrate that CB1 surface repopulation is abolished in the presence of CHX.
The ability of inverse-agonist to induce surface CB₁ upregulation 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 (Rinaldi-Carmona et al., 1998; Leterrier et al., 2004). This assumption was supported by the observation that monensin inhibited this upregulation (Leterrier et al., 2004), 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 (Mollenhauer et al., 1990). The current evidence instead suggests that inverse-agonist induced surface upregulation is a result of newly synthesized receptors being stabilized at the cell surface and therefore protected from constitutive degradation. Consistent with this hypothesis, an upregulation of total receptor number was also observed. Although this mechanism would not preclude concurrent recycling or mobilization, two crucial lines of evidence to show conclusively that cell surface upregulation with inverse-agonist treatment is due to the delivery and stabilization of newly synthesized receptors at the cell surface, and not recycling or mobilization of the intracellular pool, are presented here. Firstly, inclusion of a protein synthesis inhibitor in the assay medium completely abolished the surface upregulation, and secondly, the size of the intracellular pool did not change with inverse-agonist incubation as would be expected if CB₁ was being mobilized from the intracellular pool. The latter finding contradicts that reported by Leterrier et al. (2004), 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 utilised 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 (Rodriguez and Flemington, 1999) which could in-turn influence receptor signalling and trafficking.

Inverse-agonist upregulation and surface repopulation 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.
However, the remote possibility that CHX exerted its effects by preventing the production of a protein essential for CB₁ recycling or intracellular pool mobilization cannot be excluded.

While surface repopulation following agonist stimulation and upregulation with inverse-agonist treatment both 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 the previous assays. Putative constitutive trafficking was determined by the rate of internalisation in the absence of exogenously applied agonist. When measuring constitutive internalisation with antibody-feeding, instead of the maintenance of antibody-bound receptor in the cytoplasm as would be expected if constitutively internalised receptors were joining the intracellular pool and retaining the potential for subsequent constitutive recycling, CB₁-associated antibody was degraded. The constitutive internalisation rate correlated well with that of cell surface downregulation with CHX treatment \( (p = 0.51) \) indicating that in order to maintain a steady state, constitutively internalised receptors were replaced by newly synthesized receptors. Aside from an extremely low level of potential recycling detected in cells expressing pplss-CB₁, likely a result of gross over-expression of the receptor, no evidence for recycling following constitutive internalisation was obtained. These results therefore suggest that, while at any one time a small proportion of intracellular receptors may have recently constitutively internalised (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 (Tsao and von Zastrow, 2000a), and indeed this phenomenon has been observed for CB₁ both \textit{in vitro} (eg. Martini et al., 2007; Tappe-Theodor et al., 2007) and \textit{in vivo} (reviewed in Sim-Selley, 2003). In all cell lines studied, the application of agonist induced CB₁ degradation over a matter of hours and the effect was agonist concentration-dependent. However at the highest agonist concentrations tested, and whether long or multiple stimulations (to compensate for
potential ligand instability) were investigated, ~50% of CB₁ remained and was not degraded (compared to vehicle-treated cells). This parallels a number of in vivo studies, whereby chronic application of agonist reduced CB₁ binding by 10-50% (dependent on brain region; reviewed in Gonzalez et al., 2005). As it was confirmed that the degradative pathways were not being saturated by newly endocytosed CB₁, it appears that this residual CB₁ represents the intracellular pool and is unresponsive to agonist treatment. The only condition under which a greater proportion of cellular CB₁ was degraded was when incubated with CHX (which trended towards the eventual loss of all CB₁), indicating that although unresponsive to agonist and inverse-agonist, receptors in the intracellular pool do undergo constitutive turnover.

The body of evidence described so far strongly suggests that the intracellular pool is unresponsive to agonist or inverse-agonist stimulation. However, a recent study has suggested that the intracellular pool represents the predominant site for CB₁-mediated signalling (Rozenfeld and Devi, 2008). Although this question was not addressed directly, Gα subtypes i1, i3 and o are predominantly localised at the plasma membrane and do not colocalise with intracellular CB₁. It therefore seems unlikely that intracellular CB₁ could be signalling via archetypal G-protein pathways, although this does not discount the possibility that intracellular CB₁ might signal via non-G-protein-mediated mechanisms. While Rozenfeld and Devi demonstrate a small amount of CB₁-Gα co-immunoprecipitation, interaction of intracellular CB₁ with Gα may occur in the synthetic pathway (Dupre et al., 2006). As no surface CB₁ was detected by Rozenfeld and Devi (2008) in Neuro-2a cells, it may be that their culture had an unusual phenotype which might also contribute to these incongruous findings. Even if possible, the architecture of neuronal networks in the brain reduces the likelihood that activation of intracellular CB₁ occurs in vivo because endocannabinoids are released from activated dendrites in order to act at pre-synaptic CB₁.

While this study provides a number of important insights into CB₁ trafficking, unfortunately the exact mechanism via which the intracellular pool is formed was not identified. The inefficient
translocation and subsequent processing of CB₁ in the ER has been described previously and was correlated with CB₁’s long extracellular tail and lack of endogenous signal-sequence (Andersson et al., 2003). Similar inefficiencies in GPCR production have been reported for other receptors, such as the δ-opioid receptor whereby only approximately 40% of translated receptor is converted to the mature form, and the remaining receptor is retained in the ER and subsequently degraded (Petäjä-Repo et al., 2000). However, the intracellular receptors in this study are fully glycosylated as for the mature receptors that are delivered to the surface, and the intracellular pool is only marginally colocalised 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 slightly different mechanism must be at play. It is also interesting to note that when a pplss is chimerised to CB₁ and expressed in HEK cells, the intracellular pool is still present in similar proportions to cells expressing the wild-type receptor construct. Yet, in line with findings of Andersson et al. (2003), the overall expression level is enhanced. It also seems unlikely that the formation of CB₁’s intracellular pool might be a result of poor availability or saturation of an adaptor protein, or conversely, high levels of CB₁ expression, as the presence and general proportional size of the pool appears to be independent of expression level.

In summary, the results presented in this chapter indicate that constitutive and post-stimulatory delivery of CB₁ 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 CB₁ appears to be trafficked to a degradative pathway and does not contribute to the formation of the intracellular pool. The data suggests that this pool is instead a reservoir of receptors not delivered to the cell surface and awaiting degradation. These receptors are not re-distributed 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 suggests that these novel findings may have important implications for the design and application of cannabinoid therapeutics.
**INTRODUCTION**

Rab GTPases comprise a large family of adaptor proteins that interact with cargo both directly and via the recruitment of effector proteins to modulate a variety of processes related to intracellular protein trafficking. As detailed in chapter one, Rab proteins switch between GTP-bound “active” and GDP-bound “inactive” states that mediate functional cycling and localisation; the active forms are typically membrane-associated whereas the inactive forms are cytoplasmic. The motifs at which nucleotide binding occurs are highly conserved between Ras superfamily members and these sites can be mutated to produce Rab proteins with altered nucleotide affinity or hydrolysis efficiency (reviewed in Simpson and Jones, 2005). These mutants act in a “positive” or “negative” manner, that is, the Rab protein’s normal functions and/or interactions are enhanced or prevented. When heterologously over-expressed these mutants generally act dominantly due to competition with the endogenously expressed homologues for adaptor and effector proteins (Jung et al., 1994; Stenmark et al., 1994; Richardson et al., 1998).

The research described in chapter four strongly suggests that CB₁ does not recycle and the intracellular pool is not endocytic in origin. As already discussed, although this conclusion is in opposition to some previously published evidence, the majority of these conflicts can be explained by simple re-interpretation of data or considering the methodological limitations of the previous studies. However, some of the more difficult findings to reconcile with the current
results are the effects of Rab GTPase mutants on basal CB₁ localisation reported by Leterrier et al. (2004). This study concluded that Rab4b, generally considered to be a mediator of both slow and rapid recycling, influenced basal and ligand-induced CB₁ localisation, while Rab5, which is important for clathrin-mediated endocytosis and early endosome fusion, modulated the size of the CB₁ intracellular pool. These findings contradict those of the current study in that the size of the intracellular pool was found not to be dependent on endocytosis, and CB₁ was determined not to recycle and therefore would not be expected to interact with Rab4. Unfortunately, as the data in the Leterrier et al. paper (2004) were presented as ratios of surface to intracellular CB₁, it is not possible to determine whether changes in the ratios were due to alterations in surface or intracellular expression, or both. In addition, these experiments were quantified by manual segmentation of confocal micrographs and consequentially only a very small sample of cells were analysed (8 cells per experiment in two independent experiments) and the results could have inadvertently been influenced by human bias in image collection or segmentation.

Therefore, both in order to determine whether the present data could be reconciled with the findings of Leterrier et al. (2004) and to gain further mechanistic insight into CB₁ trafficking, the high-throughput quantitative technique described in chapter three was utilised to study the effects of Rab GTPase mutants on surface, total and intracellular CB₁. Although there are roughly 60 members of the Rab GTPase family, a small number of the most thoroughly characterised and most frequently cited Rab proteins were selected to be studied in this instance; as well as Rab4b, 5 and 11 which were studied by Leterrier et al (2004), Rab4a and 7 were also included.

As already mentioned, Rab5 is involved in endocytosis and vesicle fusion with early endosomes (Gorvel et al., 1991; Bucci et al., 1992) and this is seemingly independent of ultimate cargo protein fate (Bremnes et al., 2000). Rab7 is involved in degradative pathways, specifically the movement of cargo from early to late endosomes and late endosomes to
lysosomes (Feng et al., 1995; Bucci et al., 2000; Rink et al., 2005). Rab4 and 11 are members of the same Rab sub-family (Pereira-Leal and Seabra, 2000) and both generally considered to modulate cargo recycling; Rab11 predominantly modulates “slow” recycling via the peri-nuclear compartment (Ullrich et al., 1996; Wilcke et al., 2000), while Rab4 appears to be predominantly important for “rapid” recycling (direct from early endosomes) (van der Sluijs et al., 1992b; Yudowski et al., 2009). The human Rab4 “a” and “b” paralogs share 87% sequence identity and seem to fulfil the same functions (Simons and Zerial, 1993; Rzomp et al., 2003). However, Rab4a is phosphorylated during mitosis which leads to its accumulation in the cytoplasm and apparent inactivation, whereas Rab4b does not contain this phosphorylation site (van der Sluijs et al., 1992a; Ayad et al., 1997; Gerez et al., 2000). The differential affinity of Rab4a and b for some interacting proteins has also been noted (Bucci et al., 1999; Nagelkerken et al., 2000). Given these differences and the importance of the previous Rab4b finding in relation to CB₁ it was of interest to compare the effects of both Rab4a and b in this study. Interestingly, a handful of recent studies provide evidence to suggest that the role of Rab4 is not limited exclusively to recycling pathways but is also important for early endocytic sorting in general (van der Sluijs et al., 1992b; Cormont et al., 1996; McCaffrey et al., 2001; Hall et al., 2004; Fernandez et al., 2009).

In this chapter the effects of the aforementioned Rab GTPases on CB₁ basal expression and localisation, and post-endocytic trafficking will be investigated.

**Methods**

All experiments in this chapter were performed with the rCB₁ HEK cell line. A detailed account of the general materials and methods employed in maintaining cells and performing and quantifying experiments are provided in chapters two and three. Drug treatments are described in the text and figure legends.
To quantify or visualise intracellular receptor, cells were briefly exposed to trypsin in order to cleave surface antibody recognition sites. Anti-HA primary antibody was incubated with live cells at RT following incubation with transiently transfected protein and/or drug stimulations to detect surface CB₁, or following fixation under permeabilising conditions to detect total CB₁. Some assays utilised antibody “feeding” whereby surface CB₁ was labelled with primary antibody at 37°C prior to drug treatment. Secondary antibody was subsequently applied to live cells at RT to detect primary antibody-labelled CB₁ at the cell surface.

**Rab GTPase constructs**

The Rab4b, 5, 7 and 11 wild-type and mutant plasmid constructs were a gift from Prof. Robert Lodge (Dept. of Medical Biology, Université Laval, Quebec, Canada). Rab protein sequences were cloned from Madin-Darby Canine Kidney cell (ATCC #CCL-34) cDNA and inserted into the pEGFP-C1 plasmid (Clontech, Mountain View, CA) with EGFP adjoined to the N-terminal end of the Rab protein. The point mutations listed in Table 5.1 were generated by site-directed mutagenesis (Hunyady et al., 2002).

The Rab4a wild-type plasmid construct was a gift from Dr Marino Zerial (Max Planck Institute, Dresden, Germany). The protein sequence was cloned from human cDNA (source not stated) and inserted into the pEGFP-C3 plasmid (Clontech) with EGFP adjoined to the N-terminal end of Rab4a (Sonnichsen et al., 2000). The point mutations listed in Table 5.1 were generated by site-directed mutagenesis with a protocol slightly modified from the QuikChange® (Stratagene) approach. Briefly, 2.5 pM forward and reverse primers, 1x PfuTurbo® buffer (Stratagene), 0.2 mM dNTP mix, 2 ng/µL plasmid DNA template, 0.05 U/µL PfuTurbo® polymerase (Stratagene) and nuclease-free water were mixed in a 25uL reaction volume. This mix was subjected to temperature cycling as in Table 2.1 in a Palm-Cycler PCR machine (Corbett Life Science, Concorde, NSW, Australia). The PCR reaction was then incubated with 0.375 U/µL DpnI (Promega, Madison, WI) at 37°C for 60 min to digest the dam-methylated template DNA and leave only the PCR-amplified mutated product. Primers were designed according to the
QuikChange® recommendations; forward primer sequences were as follows (mutagenic residues are underlined; reverse-complement sequences were also utilised):

Q67L: CAAATATGGGATACAGCAGGACTAGAACGATTCAGG
S22N: GGAAATGCAGGAACTGGCAAAATTGCTTACTTCATCAG

Mutations were analogous to those for 4b and have been utilised in a number of previous studies (eg. Cormont et al., 1996; Nagelkerken et al., 2000; McCaffrey et al., 2001).

Plasmids were transformed into XL10-Gold competent bacteria and amplified by midiprep. Prior to use in experiments sample DNA concentrations were quantified and sequences verified (see also pg. 27).

<table>
<thead>
<tr>
<th>Rab protein</th>
<th>Originating species, isoform amino acid homology with <em>Homo sapiens</em></th>
<th>“Positive” (GTP-bound) mutation</th>
<th>“Negative” (GDP-bound) mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td><em>Homo sapiens</em>, 100%</td>
<td>Q67L</td>
<td>S22N</td>
</tr>
<tr>
<td>4b</td>
<td><em>Canis lupus familiaris</em>, 100%</td>
<td>Q67L</td>
<td>S22N</td>
</tr>
<tr>
<td>5a</td>
<td><em>Canis lupus familiaris</em>, 99.1%</td>
<td>Q79L</td>
<td>S34N</td>
</tr>
<tr>
<td>7a</td>
<td><em>Canis lupus familiaris</em>, 99.5%</td>
<td>Q67L</td>
<td>N125I</td>
</tr>
<tr>
<td>11a</td>
<td><em>Canis lupus familiaris</em>, 100%</td>
<td>Q70L</td>
<td>S25N</td>
</tr>
</tbody>
</table>

Table 5.1 Rab GTPase isoforms and point-mutants used in this study
### Transient transfection

Based on protocols provided by the transfection reagent manufacturer (Ciccarone et al., 1999; Pichet and Ciccarone, 1999), three transient transfection methods were piloted. For all methods, 2 µL Lipofectamine™ 2000 per 100 µL Opti-MEM® was mixed and incubated at RT for 5 min. DNA was mixed with Opti-MEM® at 0-8 ng/µL; 5 ng/µL was used for the majority of experiments. The Lipofectamine™ 2000 and DNA mixes were then combined in equal volumes and incubated at RT for 20 min prior to application to cells, as noted below. Unless indicated, cells were seeded on Poly-L-Lysine or Poly-D-Lysine-treated plates or slides as appropriate for the experiment.

**a) Transfect and re-plate cells**

Cells were seeded in a 24-well (untreated) plate at an appropriate density to reach 90-100% confluence by the time of tranfection and allowed to recover overnight. The following day 200 µL of the Lipofectamine™/DNA mix per well was added drop-wise. Approximately 24 h later cells were trypsinised and re-plated at a density expected to reach 70-80% confluence by

<table>
<thead>
<tr>
<th>Description</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
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<tbody>
<tr>
<td>Denaturation</td>
<td>1</td>
<td>95°C</td>
<td>60 s</td>
</tr>
<tr>
<td>Denaturation</td>
<td>16</td>
<td>95°C</td>
<td>30 s</td>
</tr>
<tr>
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<td>55°C</td>
<td>60 s</td>
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<td>Extension</td>
<td></td>
<td>68°C</td>
<td>6 min 18 s</td>
</tr>
<tr>
<td>Extension</td>
<td>1</td>
<td>68°C</td>
<td>2 min</td>
</tr>
<tr>
<td>(End)</td>
<td>1</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

Table 5.2 PCR cycling conditions for site-directed mutagenesis
the end of the experiment. Approximately 10-20% greater plating densities than would usually be used for un-transfected cells were required.

b) Transfect and plate cells simultaneously

50 µL Lipofectamine™/DNA mix per well was dispensed into a 96-well plate. Cells were seeded on top of the transfection mix at a density expected to reach 70-80% confluence by the end of the experiment. Approximately twice the number of cells that would usually be used when plating un-transfected cells were required. Cells were seeded in double the usual volume (150 µL) of full-serum media as using this larger volume improved apparent cell health and survival following transfection.

c) Plate cells then transfect

Cells were plated at a density expected to reach 60-70% confluence approximately 24 h later for 18 h and 24 h post-transfection timepoints, or 50-60% confluence for a 48 h post-transfection timepoint. When at the desired confluency, the Lipofectamine™/DNA mix was gently added to cells. 50 µL mix per well was used in a 96-well plate or 200 µL mix per well in an 8-well divided slide.

CB1 and Rab GTPase quantification

Following image acquisition with Discovery-1™, CB1 and EGFP/Rab expression was quantified in MetaMorph® with the TGVC method described in chapter three. Mean Fluorescence Ratios (MFR) were calculated by dividing surface by intracellular CB1. In order to mimic the analysis method utilised by Leterrier et al (2004), in each experiment values for surface CB1 were normalised to total minus intracellular CB1 expression in EGFP-transfected cells, while intracellular CB1 was normalised to total CB1 expression in EGFP-transfected cells. That is, for the EGFP-transfected control, the sum of the normalised values for surface and intracellular CB1 would equal 100%. Thereby, normalised values for surface and intracellular CB1 were comparable as though they had been captured in the same images and undergone the same
immunocytochemistry. A second, theoretically equivalent, equation was also considered: the inverse of the intracellular pool minus one (derivation of this equation is provided in the appendix, pg. 180). This equation produced close to identical overall results but the first described equation was ultimately used as it was considered closer to the method of Leterrier et al. (2004) and more appropriate to use the directly measured surface and intracellular CB₁ values in calculating the MFRs.

Transfection efficiency (ie. the percentage of EGFP/Rab-positive cells), was quantified with the “Cell Scoring” MetaMorph® drop-in assay (see also Scotter et al., 2008). This assay identifies areas of signal above local background for the protein of interest (ie. EGFP) and correlates these areas with the position of Hoechst-stained cell nuclei identified with the same algorithm as “Count Nuclei” (pg. 47) to produce counts of nuclei that are positive or negative for the protein of interest. User-set parameters define the approximate minimum and maximum width of nuclei and cells and the typical difference in intensity of fluorescence compared to background. The positive cell count was divided by the total nuclei count to calculate the percentage of positive cells in each image.

RESULTS

Optimisation of transient transfection

A multitude of prior studies utilising transient transfection to heterologously over-express a protein of interest exist, and in turn many protocols are available. As this technique was not previously established in the laboratory where this research was carried out, transfection conditions were tested and optimised at the initiation of this set of experiments. Ideally, the method would maximise transfection efficiency (ie. the proportion of cells successfully transfected), construct expression and cell health and viability. As listed in the methods (pg. 120), three main approaches were investigated. Empty EGFP vector was utilised in the optimisation. Lipofectamine™ 2000, a popular reagent for mammalian cell transfection which
acts by forming cationic liposome complexes with DNA, was used throughout these experiments.

Using the starting Lipofectamine™ to DNA ratio recommended by the manufacturer, the transfection efficiency for EGFP, and counts of cells exposed to Lipofectamine™ with or without plasmid DNA, were compared for the three transfection methods at 48h post-transfection (Figure 5.1A). The greatest transfection efficiency was achieved with method (b), transfect and plate cells simultaneously, with an average of 89.3% EGFP-positive cells (± 3.9%). Method (c), plate cell then transfect, yielded a slightly but not significantly lower efficiency (81.0 ± 4.1%, \( p = 0.36 \)), while method (a), transfect then plate cells, produced the lowest efficiency (44.5 ± 9.1%). It was noted in pilot experiments that larger numbers of cells needed to be plated to reach the desired confluency by the end of the experiment than would be expected for non-transfected cells. Although it is not clear whether this was mediated by reduced cell proliferation, reduced cell adhesion or increased cell death, this appeared to be a result of DNA transfection because cells exposed to Lipofectamine™ alone were qualitatively no different in morphology or confluency to untreated cells. Altered cell viability or proliferative capacity was particularly noticeable for method (b) for which twice the usual number of cells were required to be plated and a relatively large difference in the final cell density at the end of the experiment compared to un-transfected cells (exposed to Lipofectamine™ without DNA) was noted (62.9 ± 6.8%). The final density of cells transfected by methods (a) and (c) were not significantly different to un-transfected cells, however the cell viability for method (a) was particularly variable between experiments. Qualitatively, cells appeared essentially normal following transfection with method (c), whereas some unusual cell morphology and an increase in cellular debris were noted with methods (a) and (b). So as to reduce the likelihood that cellular processes would be altered non-specifically by the act of transient transfection, method (c) was selected as the preferred method.
Although a 48 h post-transfection timepoint has been used in a number of Rab protein studies (e.g. Leterrier et al., 2004; Yudowski et al., 2009), by this timepoint cells had a tendency to clump together rather than grow in a continuous monolayer which is not desirable for quantification of cell number and may in itself influence cellular functions. Further, the longer mutant Rab proteins were expressed in cells the greater the likelihood that the dominant-negative or positive mutants might have increasing cumulative deleterious effects on general cellular functions and potentially influence the protein of interest (CB₁) through non-specific mechanisms. A range of transfection times were therefore tested. As shown in Figure 5.1B, the percentage of EGFP-positive cells increased between 6 and 18 h and reached a plateau between 18 and 48 h. Relative to non-transfected cells, the transfected cell count reduced from 90.6 ± 1.9% at 18 h post-transfection to 75.4 ± 3.7% by 48 h. As well as exhibiting a high transfection efficiency and a similar cell density to un-transfected cells, cells at the 18 h timepoint had better morphology and appeared healthier than cells incubated for longer times. This time point was therefore selected for use in subsequent experiments.

Finally, the DNA concentration was optimised while keeping the Lipofectamine™ concentration constant. The percentage of EGFP-positive cells and the level of EGFP expression were tested following transfection with a range of EGFP plasmid concentrations. Note that EGFP expression was only averaged between EGFP-positive cells and therefore is reflective of EGFP expression within the transfected population. The transfection efficiency appeared to plateau at near-to 75% when transfecting with more than 4 ng/uL, whereas the EGFP expression reached a maximum between 4 and 6 ng/uL but dropped at higher concentrations, perhaps as a result of reduced cell health due to protein over-expression or a sub-optimal ratio of Lipofectamine™ to DNA (Figure 5.1C). Subsequent Rab protein co-expression experiments in this chapter utilise method (c) to transfect HEK rCB₁ cells with 5 ng/µL DNA (in the initial DNA-Opti-MEM® dilution) and were terminated 18 h post-transfection.
All the transfection techniques piloted produced a wide range of introduced construct expression levels. This is demonstrated in Figure 5.2A; at an appropriate image brightness for the highest expressing cells, low to medium-expressing cells are difficult to detect, whereas at an image brightness appropriate for cells with lower EGFP levels the highly expressing cells produce an over-saturated signal. The majority of EGFP-Rab images presented in Figure 5.3 to Figure 5.7 are at levels that allow ready detection of low levels of EGFP which has resulted in saturation of the signal arising from some cells.

Although some previous studies have observed effects of wild-type Rab GTPase over-expression on receptors (eg. Seachrist et al., 2000; McCaffrey et al., 2001), none of the Rab subtypes tested produced significant changes in CB₁ surface or total expression as compared with cells transiently expressing EGFP alone ($p = 0.16$ and 0.79 respectively; Figure 5.2B). In subsequent experiments investigating the effects of the Rab GTPase dominant-positive and negative mutants, results were normalised to the measure of interest for cells transfected with the wild-type Rab protein. Interestingly, cells only exposed to Lipofectamine™ 2000 without DNA exhibited higher levels of surface and total CB₁ compared with EGFP-transfected cells ($p = 0.050$ [near statistical significance] and 0.0023 respectively).
Figure 5.1 Optimisation of transient transfection protocol

(A) Comparison of EGFP transfection efficiency (“positive cells”) and cell counts (normalised to density of non-transfected cells) for three transfection methods 48 h post-transfection with 8 ng/µL DNA. Methods were: (a) transfect and re-plate cells, (b) transfect and plate cells simultaneously and (c) plate cells then transfect. Significance comparisons are as indicated for efficiency and with non-transfected cells (100%) for cell count. (B) EGFP transfection efficiency (“positive cells”) and cell counts (normalised to density of non-transfected cells) with transfection method (c) at various times following transfection with 8 ng/µL DNA. (C) EGFP transfection efficiency (“positive cells”) and average expression 18 h post-transfection with method (c) and varying DNA concentrations. Expression was averaged between only the EGFP-positive cells and normalised to the maximum observed expression level in each experiment. DNA concentrations refer to the initial DNA/OptiMEM® mix prior to combination with Lipofectamine™. Arrow indicates DNA concentration selected for subsequent experiments.
Figure 5.2 Demonstration of transiently-transfected EGFP expression variability and effect of wild-type Rab GTPase expression on CB₁.

(A) Images of EGFP expression at two brightness levels (“Low”, “High”) demonstrating variability in construct expression when transiently transfected and associated Hoechst staining (“Nuclei”). EGFP-negative cells marked with (–), EGFP-positive cells marked with (+). Bar, 10 µm. (B) Surface and total CB₁ expression in cells exposed to Lipofectamine™ 2000 without DNA (“nD”) or transfected with wild-type EGFP-Rabs. Data is normalised to surface or total CB₁ expression in cells transfected with EGFP empty vector (100%).
Rab GTPase modulation of basal CB₁ expression

The results in this section are grouped by the generally accepted Rab GTPase functions: Rab5 (internalisation), Rab4a, 4b and 11 (recycling) and Rab7 (degradation). CB₁ surface, intracellular and total expression, as well as the MFR, were measured for each Rab mutant and normalised to the equivalent measure for the wild-type Rab. Note that the GTP-bound (positive) mutants are expected to be membrane bound, whereas GDP-bound (negative) forms are expected to be cytoplasmic (Feng et al., 2001), although over-expression may result in the saturation of interactions with endogenous proteins producing some diffuse cytoplasmic and nuclear localisation of the wild-type and positive mutants (Gorvel et al., 1991; Pereira-Leal et al., 2001; Gomes et al., 2003).

Rab5 (Internalisation)

As a mediator of clathrin-dependent endocytosis and early endosome fusion, over-expression of the Rab5 dominant-positive mutant was expected to increase constitutive internalisation and thereby decrease CB₁ basal surface expression, whereas the dominant-negative mutant was expected to inhibit constitutive internalisation and thereby increase basal surface expression.

Unexpectedly, the Rab5 dominant-positive mutant increased CB₁ surface expression (141.4 ± 11.2%, \(p = 0.0059\)), however intracellular CB₁ also trended towards being increased (209.7 ± 45.7%, \(p = 0.059\) [close to significance]; Figure 5.3A). Concomitantly, total CB₁ was also increased (181.3 ± 32.5%, \(p = 0.0025\)). The slightly greater increase in intracellular relative to surface CB₁ resulted in a trend towards a decreased MFR (74.5 ± 10.9%, \(p = 0.47\)). When visualised (Figure 5.3B-C), Rab5 and CB₁ were colocalised in very large endosomal structures. These striking features closely resemble the “giant early endosomes” commonly observed when the Rab5 Q79L mutant is over-expressed and assumed to be a result of stimulated homotypic early endosome fusion (Seachrist et al., 2000; Volpicelli et al., 2001; Stenmark et al., 1994; Hoffenberg et al., 1995). These structures appear to have trapped constitutively internalised CB₁ and resulted in the quantified increase in intracellular CB₁.
The dominant-negative mutant acted as expected, producing a significant increase in surface CB$_1$ (162.3 ± 17.8%, $p = 0.0011$) and total CB$_1$ expression (126.3 ± 9.15%, $p = 0.018$). There was no evidence to suggest that the size of the intracellular pool was influenced by the dominant-negative mutant expression (97.2 ± 4.45%, $p = 0.99$). The elevated surface expression without a change in intracellular CB$_1$ resulted in an increased MFR compared to cells expressing wild-type Rab5 (169.0 ± 22.9%, $p = 0.0075$). In agreement with the quantification, when visualised the presence of the dominant-negative mutant did not appear to influence cytoplasmic CB$_1$ localisation, although an increase in surface expression was evident.
Figure 5.3 Basal CB₁ with Rab5 co-expression

(A) Surface, total and intracellular CB₁ expression, and CB₁ MFR, with dominant-positive (“P”) and negative (“N”) Rab5 transient expression, relative to CB₁ expression or MFR in cells transfected with wild-type Rab5 (100%). (B-C) Widefield (B) and confocal (C) images of total CB₁ (red) co-expressed with dominant-positive (“P”), wild-type (“W”) or dominant-negative (“N”) Rab5 (green). Bars, (B) 10 µm; (C) 5 µm.
CHAPTER FIVE

RAB GTPASE MODULATION OF CB₁ TRAFFICKING

A

CB₁ Expression or MFR (% wt Rab5)

0 25 50 75 100 125 150 175 200 225 250

Surface Intracellular Total MFR

P N P N P N P N P N

* **

B

CB₁  Rab5  Merge

P

W

N

C

P  W  N
Rabs 4a, 4b and 11 (Recycling)

As already mentioned, Rabs 4a, 4b and 11 are all generally considered to mediate post-endocytic recycling and Rab4 may also have a more general role in early endosomal sorting. Although a previous study observed effects of Rab4b on basal CB$_1$ localisation (Leterrier et al., 2004), based on the results presented in chapter four indicating that CB$_1$ does not undergo post-endocytic recycling, over-expressing mutants of these Rab types was not expected to influence CB$_1$ localisation.

In line with these expectations, no evidence to suggest that over-expression of dominant-positive or negative Rab4a, 4b or 11 influenced basal CB$_1$ expression was obtained ($p = 0.53$, $p = 0.87$, $p = 0.51$ respectively; Figure 5.4 - Figure 5.6). Interestingly, the dominant-positive mutants of both Rab4a and b produced trends towards increased intracellular CB$_1$ (a: 113.4 ± 7.5%; b: 122.8 ± 12.9%) which were associated with trends towards increased total CB$_1$ expression and reduced MFRs. This slight increase in intracellular CB$_1$ was also evident when cells expressing the Rab4a and b dominant-positive mutants were visualised (Figure 5.4B-C, Figure 5.5B-C). CB$_1$ appeared in enlarged vesicles and was partially colocalised with Rab4. No remarkable changes to CB$_1$ localisation with Rab11 expression were observed. Although intracellular pool CB$_1$ localised to a similar area of the cytoplasm as the Rab11 wild-type and dominant-positive forms, little direct colocalisation was observed by confocal microscopy (Figure 5.6B-C).
Figure 5.4 Basal CB₁ with Rab4a co-expression

(A) Surface, total and intracellular CB₁ expression, and CB₁ MFR, with dominant-positive (“P”) and negative (“N”) Rab4a transient expression, relative to CB₁ expression or MFR in cells transfected with wild-type Rab4a (100%). (B-C) Widefield (B) and confocal (C) images of total CB₁ (red) co-expressed with dominant-positive (“P”), wild-type (“W”) or dominant-negative (“N”) Rab4a (green). Bars, (B) 10 μm; (C) 5 μm.
CHAPTER FIVE

RAB GTPASE MODULATION OF CB₁, TRAFFICKING

A

CB₁ Expression or MFR (% wt Rab4a)

Surface
Intracellular
Total
MFR

0 25 50 75 100 125

P N P N P N P N

B

CB₁ Rab4a Merge

P

W

N

C

P W N
Figure 5.5 Basal CB₁ with Rab4b co-expression

(A) Surface, total and intracellular CB₁ expression, and CB₁ MFR, with dominant-positive (“P”) and negative (“N”) Rab4b transient expression, relative to CB₁ expression or MFR in cells transfected with wild-type Rab4b (100%). (B-C) Widefield (B) and confocal (C) images of total CB₁ (red) co-expressed with dominant-positive (“P”), wild-type (“W”) or dominant-negative (“N”) Rab4b (green). Bars, (B) 10 µm; (C) 5 µm.
CHAPTER FIVE  
RAB GTPASE MODULATION OF CB₁ TRAFFICKING

A

![Graph showing CB₁ expression or MFR (µM Rab4b)]

- Surface
- Intracellular
- Total
- MFR

B

![Immunofluorescence images of CB₁ and Rab4b]

CB₁ | Rab4b | Merge
---|---|---
P | ![Imaging of P condition] | ![Imaging of P condition] | ![Imaging of P condition]
W | ![Imaging of W condition] | ![Imaging of W condition] | ![Imaging of W condition]
N | ![Imaging of N condition] | ![Imaging of N condition] | ![Imaging of N condition]

C

![Imaging of additional conditions]

P | ![Imaging of additional P condition] | ![Imaging of additional P condition] | ![Imaging of additional P condition]
W | ![Imaging of additional W condition] | ![Imaging of additional W condition] | ![Imaging of additional W condition]
N | ![Imaging of additional N condition] | ![Imaging of additional N condition] | ![Imaging of additional N condition]

Scale bars: 2 µm.
Figure 5.6 Basal CB1 with Rab11 co-expression

(A) Surface, total and intracellular CB1 expression, and CB1 MFR, with dominant-positive (“P”) and negative (“N”) Rab11 transient expression, relative to CB1 expression or MFR in cells transfected with wild-type Rab11 (100%). (B-C) Widefield (B) and confocal (C) images of total CB1 (red) co-expressed with dominant-positive (“P”), wild-type (“W”) or dominant-negative (“N”) Rab11 (green). Bars, (B) 10 µm; (C) 5 µm.
Rab7 (Degradation)

Rab7a has been shown to mediate early to late endosome maturation and trafficking from late endosomes to lysosomes. As it was observed in chapter four that CB₁ has a degradative phenotype following both agonist-induced and constitutive endocytosis, mutants were expected to alter total CB₁ expression by blocking (dominant-negative) or enhancing (dominant-positive) CB₁ constitutive degradation.

Unexpectedly, total CB₁ did not markedly increase with expression of the Rab7 dominant-negative mutant, indicating that basal CB₁ degradation was not blocked. A significant increase in surface CB₁ was noted, however (113.5 ± 4.9%, \( p = 0.015 \); Figure 5.7A). This small change in surface CB₁ resulted in a trend towards an increased MFR (125.0 ± 15.1%, \( p = 0.10 \)).

Expression of the dominant-positive mutant increased intracellular CB₁ (130.1 ± 7.2%, \( p = 0.045 \)) and the proteins were well colocalised by widefield microscopy (Figure 5.7B). Interestingly, closer inspection by confocal imaging revealed that for both the dominant-positive mutant and wild-type constructs, although regionally colocalised, areas of strong CB₁ staining were often Rab7-negative and vice versa (Figure 5.7C).
Figure 5.7 Basal CB₁ with Rab7 co-expression

(A) Surface, total and intracellular CB₁ expression, and CB₁ MFR, with dominant-positive (“P”) and negative (“N”) Rab7 transient expression, relative to CB₁ expression or MFR in cells transfected with wild-type Rab7 (100%).  (B-C) Widefield (B) and confocal (C) images of total CB₁ (red) co-expressed with dominant-positive (“P”), wild-type (“W”) or dominant-negative (“N”) Rab7 (green). Bars, (B) 10 μm; (C) 5 μm.
CHAPTER FIVE

RAB GTPase MODULATION OF CB₁ TRAFFICKING

A

CB₁ Expression or MFR (% wt Rab7)

Surface Intracellular Total MFR

P N P N P N P N

0 25 50 75 100 125 150

B

CB₁ Rab7 Merge

P

W

N

C

P W N
Rab GTPases do not influence inverse agonist-induced surface upregulation or recycling following agonist stimulation

As the majority of the Rab GTPase mutants studied exerted little influence on basal CB₁ expression, particularly surface expression, two ligand-mediated trafficking assays were investigated in the presence of Rab mutant proteins to determine whether any Rab-mediated effects would be revealed or amplified.

SR, a CB₁ inverse-agonist, was determined in chapter four to induce upregulation of surface CB₁ by preventing constitutive internalisation and stabilising newly synthesised receptors at the cell surface. When compared to SR-induced surface CB₁ upregulation in cells expressing wild-type Rab protein, none of the Rab mutants tested (4a, 4b, 11 and 7) produced significant changes in the extent of upregulation after a 4 h treatment \( (p = 0.059; \text{Figure 5.8A}) \). A trend toward greater upregulation was noted with the Rab7 dominant-negative mutant \((116.1 \pm 9.1\%)\), reflecting the effect observed in the basal CB₁ localisation study (pg. 139).

One of the experiments presented in chapter four that contributed evidence to suggest that CB₁ does not recycle was the antibody “live feeding” assay whereby primary antibody-labelled CB₁ internalised, however did not recycle back to the cell surface following washing and incubation with inverse-agonist (pg. 91). This experiment was repeated in cells transiently transfected with the Rab wild-type and mutant proteins to determine whether the presence of the Rab proteins would switch the post-endocytic fate of CB₁ to a recycling pathway. As demonstrated in Figure 5.8B, performance of the antibody live-feeding experiment in the presence of over-expressed Rab protein did not provide any evidence to suggest that CB₁ recycled following agonist-induced internalisation.
Figure 5.8 Rab GTPase influence on SR-induced CB₁ surface upregulation and recycling with live antibody feeding

(A) Extent of 1 µM SR-induced CB₁ surface upregulation in the presence of Rab dominant-positive ("P") and negative ("N") mutant proteins following 4 h treatment, relative to the extent of upregulation in cells expressing the matched wild-type Rab (100%).  

(B) Antibody “live-feeding” experiment to measure bona fide CB₁ recycling.  Surface CB₁ was labelled with primary antibody then internalised with 10 nM HU (15 min, “internalised”), washed, then incubated with 1 µM SR for 5 h (“recycled”).  Cells were transfected with Rab dominant-positive mutant ("P"), wild-type ("W"), or dominant-negative mutants ("N").  Secondary antibody was applied under non-permeabilising conditions to detect primary antibody-bound surface receptor.  Results are normalised to basal surface CB₁ in cells expressing the same Rab construct (100%).
**DISCUSSION**

In this chapter a protocol for transient expression of a heterologously introduced plasmid construct in HEK cells was optimised and applied to study the effects of selected Rab GTPases on CB1 expression and trafficking.

As transient transfection is known to be deleterious to the recipient cells (Rodriguez and Flemington, 1999), care was taken when optimising the transfection conditions to ensure that, as well as robust Rab construct expression, cell viability was maximised. Indeed, reduced cell numbers compared to un-transfected cells were observed with all methods. This was likely due to reduced cell proliferation, adhesion and/or viability as a result of competition between endogenous genes and the introduced constructs for transcription and/or translation machinery. Method (c), whereby cells were transfected in the assay plate after recovery from seeding, was selected as the preferred protocol because this produced the best apparent cell health and near-maximal transfection efficiency compared with the other two methods tested. The most appropriate timepoint following transfection at which to measure Rab effects on CB1 was considered next. It was anticipated that prolonged expression of very high levels of heterologous protein may have deleterious effects on normal cell function that could influence CB1 trafficking non-specifically. In particular, the potential effects of long-term Rab mutant expression were difficult to predict. Indeed, it was noted in pilot experiments that 48 h post-transfection fewer cells were present in wells transfected with the Rab11 dominant-negative mutant compared with wild-type Rab11 (77.7 ± 5.6%, $p = 0.029$), indicating that this isoform had negatively influenced cell viability or proliferation. Ultimately an 18 h timepoint post-transfection was selected as very little difference in cell densities between transfected and un-transfected cells was noted (including for the Rab11 negative mutant; 104.6 ± 5.5%, $p = 0.49$), and near-to maximal transfection efficiency was maintained. Finally, the DNA concentration for transfection was optimised; the concentration selected was lower than suggested by the manufacturer but produced good transfection efficiency and construct expression.
Although some studies have reported effects on the protein of interest as a result of wild-type Rab protein over-expression (e.g. Seachrist et al., 2000; McCaffrey et al., 2001), none were evident in this study. It was therefore considered appropriate to present the results of the Rab mutant experiments as normalised to the wild-type isoform of the same Rab. Interestingly, a marked reduction in average cellular CB₁ expression was noted following transient transfection with all the methods and constructs tested. It is unlikely that this was a consequence of the specific construct expressed as both EGFP and the Rab constructs produced the same effect, nor was this an effect of exposure to Lipofectamine™ 2000 (data not shown). As for the observed influence of transfection on cell health and proliferation, the overall reduction in CB₁ expression following transfection may have been a result of competition between CB₁ (and perhaps other cellular proteins) and the transfected construct for protein synthesis machinery. It is unlikely that the reduction in CB₁ expression resulted from direct competition for promoter-specific regulators because the CB₁ and Rab constructs were under the control of different constitutive promoters (Human elongation factor 1α and Human cytomegalovirus respectively).

In future it may be useful to perform the transient transfection in the presence of selection pressure antibiotics to promote continued robust CB₁ expression. To prevent this phenomenon influencing the results of this set of experiments, care was taken to ensure cells were transfected with equivalent amounts of DNA between Rab constructs. Namely, the concentrations of DNA preparations were taken as the average of multiple spectrophotometer readings and two independent DNA preparations were used over the course of this study. With the exception of the Rab7 negative mutant (discussed further below), equivalent transfection efficiencies were achieved for all the Rab constructs ($p = 0.65$).

The regulatory capacity of Rab proteins is dependent on cycling between the GTP-bound “active” and GDP-bound “inactive” states, that is, GTP hydrolysis is not required for Rab function per se and instead appears to be important for termination of Rab function and/or membrane association (Barbieri et al., 1994; Rybin et al., 1996; Richardson et al., 1998). Capitalising on these properties, point mutations can be introduced that lock the Rab GTPases
in one or other state. Due to the conservation of nucleotide binding motifs, equivalent
mutations can be applied to different members of the Rab family to confer the same effect. In
this study the dominant-positive mutants were generated by switching glutamine for leucine in
the WDTAGQE motif (in the region of amino acid 67 – 79), while the majority of the negative
forms were serine to asparagine mutants in the GKS motif (in the region of amino acid 22 – 34).
The former mutation reduces native and GTPase activating protein (GAP)-mediated GTP
hydrolysis, thereby creating a constitutively active Rab protein, while the serine to asparagine
point mutation greatly reduces the nucleotide binding site affinity for GTP and therefore the
resultant protein predominantly exists in a GDP-bound inactive state (Der et al., 1986; Feig and
Cooper, 1988; Walworth et al., 1992; Hoffenberg et al., 1995). The Rab7 dominant-negative
phenotype was produced with an asparagine to isoleucine mutation at amino acid 125 in the
NKXD region which also confers a greatly reduced affinity for both GTP and GDP (Sigal et al.,
1986; Pai et al., 1989; Gorvel et al., 1991; Hoffenberg et al., 1995). When transiently
expressed in HEK cells, the wild-type and mutant Rab proteins generally appeared as expected
with the wild-type and dominant-positive isoforms membrane-bound and dominant-negative
isoforms diffusely cytoplasmic. Some diffuse cytoplasmic and occasionally nuclear expression
was also noted for the wild-type and positive mutants which was likely a symptom of over-
expression and consequential saturation of the membrane association mechanisms (Gorvel et
al., 1991; Pereira-Leal et al., 2001; Gomes et al., 2003).

Based on Rab5’s known role in endocytosis and early endosome fusion and the results of
previous studies with GPCRs (eg. Seachrist et al., 2000; Volpicelli et al., 2001), the dominant-
negative mutant was expected to prevent constitutive internalisation and thereby increase basal
surface CB₁ expression, while the dominant-positive mutant was expected to decrease basal
surface expression. The negative mutant did indeed increase surface CB₁ while the size of the
intracellular pool was unchanged, further corroborating the data presented in chapter four
suggesting that the intracellular pool is not formed by constitutively internalising receptors.
Unexpectedly, introduction of the dominant-positive mutant elevated both surface and intracellular CB1 expression. On inspection of the cells, however, it was immediately evident that the cytoplasmic CB1 had a strikingly different localisation to that usually observed and that CB1 and Rab5 were colocalised in enlarged endosomal structures. These appeared to be giant early endosomes, the formation of which is a widely reported consequence of Rab5 dominant-positive mutant expression (e.g., Seachrist et al., 2000; Volpicelli et al., 2001) and is believed to be a result of stimulated early endosome fusion (Stenmark et al., 1994; Hoffenberg et al., 1995). In addition, interaction of the Rab5 dominant-positive mutant appears to lock endocytosed cargo in these endosomes thereby preventing subsequent trafficking and/or endosomal maturation (Stenmark et al., 1994). Thereby the observed increase in cytoplasmic CB1 likely resulted from blocked degradation of constitutively endocytosed CB1. Detailed ultrastructural analysis has revealed that giant early endosomes are in fact multi-vesicular bodies. A correlation has been drawn whereby cargo at the surface of the structures would normally recycle, however this is inhibited by the mutated Rab protein. Whereas cargo appearing on the inside of the multi-vesicular body would normally be degraded (Volpicelli et al., 2001). CB1 is predominantly localised to the interior of the giant early endosomes, indicating that constitutively endocytosed CB1 is usually destined for degradation and paralleling the conclusions set out in chapter four.

Although the dominant-positive Rab5 mutant is usually reported to increase endocytosis and would thereby be expected to decrease basal surface expression for constitutively active receptors, in this instance the mutant appears to have acted in a somewhat dominant-negative manner with respect to constitutive endocytosis, resulting in surface upregulation. As constitutively active Rab5 has reduced GTPase activity and may therefore be limited in its ability to initiate new rounds of transport (Stenmark et al., 1994; Lafourcade et al., 2004), Rab5 itself may become sequestered in the giant early endosomes, perhaps along with limiting adaptor proteins, and consequentially produce an endocytosis deficit. Conceivably, the relatively rapid rate of CB1 constitutive internalisation exaggerates this effect in comparison with
other receptors studied thus far. However the calculated MFR, that is surface expression divided by intracellular, trended towards being reduced relative to wild-type Rab5, indicating that cytoplasmic CB1 increased to a greater extent than surface CB1 and the net effect of the Rab5 positive mutant expression was enhanced constitutive endocytosis.

Both this MFR result and that for the dominant-negative mutant are in agreement with the results of Leterrier et al (2004) and confirm that Rab5 plays a pivotal role in CB1 endocytosis. However in the previous report, the alterations in MFR were attributed to intracellular pool mobilisation; in particular, that blockade of endocytosis by the dominant-negative mutant facilitated re-distribution of cytoplasmic CB1 to the cell surface. By monitoring surface and intracellular CB1 expression separately, the present study reveals that this is not the case and demonstrates the care that must be taken when interpreting indirect ratiometric measurements.

Unlike the Rab5 results, the Rab4 mutant-induced alterations in the CB1 surface to intracellular ratios were not analogous to the results of Leterrier et al. (2004). The 2004 study reported an increase in the MFR with the Rab4b dominant-positive mutant and the opposite for the negative mutant, and concluded that these results were consistent with the theory that CB1 undergoes Rab4-dependent constitutive recycling. However here, the Rab4 negative mutants exerted no apparent effects and expression of the positive mutants led to marginal reductions in the CB1 MFRs as a result of slightly increased intracellular CB1. In agreement with the results of Leterrier et al. (2004), Rab11 and its mutant forms were not observed to influence basal CB1 localisation, delivery to the surface or recycling.

The reasons for the disparity in the Rab4 results between the two studies under comparison are not clear. While some methodological differences can be identified, namely that in the Leterrier et al. (2004) study a transfection protocol similar to method (a), transfect then re-plate cells, was used; both the Rab constructs and untagged rCB1 were transiently co-transfected (versus stable transfect of HA-rCB1 in this study); and a 48 h post-transfection assay point was
used (versus 18 h in this study), the similarity of results obtained for Rab5 and Rab11 seem to reduce the likelihood that the disparity in Rab4 results can easily be attributed to these factors. Furthermore, the EGFP-Rab5, 4b and 11 wild-type and mutant plasmids were obtained from the same source and HEK-293 cells were utilised for both studies. As blinding is not mentioned in the Leterrier et al. (2004) report, one possibility in terms of this discrepancy being artefactual is that the results were unintentionally biased by the researcher carrying out imaging and analysis because of their expectations based on working hypotheses. In addition, the article does not conform to generally accepted standards (Cumming et al., 2007) in that the experiment was only repeated two independent times and results were presented as the standard error of the data from a single experiment of eight cells (ie. the standard error of one sample, instead of multiple sample means). It is therefore not possible to garner an indication of error between independent experiments from the data presented. Despite this, the possibility that both studies are correct should not be discounted. It would certainly be interesting to bring together the exact cell and CB1 construct samples used in the two studies and perform the experiments under the same conditions to determine whether the difference between the two studies can be replicated. If so, this may assist in determining the mechanisms by which CB1 trafficking determination to different pathways. However, in chapter four a range of experiments suggesting that CB1 does not recycle were corroborated in four cell lines, one with endogenous CB1 and the others heterologously expressing two different receptor constructs. It is therefore highly likely that CB1’s default phenotype is degradative and non-recycling.

As, under the conditions tested in this study, the Rab4a and b dominant-negative mutants exerted no apparent effect on CB1 localisation, it seems that Rab4 does not mediate CB1 trafficking directly. The increase in cytoplasmic CB1 that occurred with expression of the dominant-positive mutant was unexpected but may have arisen from inhibition of early endosome maturation via similar mechanisms to those hypothesised with the Rab5 GTP-locked mutant. Although an increase in cytoplasmic CB1 would also be anticipated if the GTP-locked
Rab4 had exerted its effect via blockade of constitutive recycling, an inverse effect on surface expression would be expected. As no such change was observed it seems the effect is due to inhibition of post-endocytic pathways. Combined with the pictorial evidence that the Rab4 positive mutant seemed to produce slight enlargement of early endosomes, it seems that Rab4-GTP is able to indirectly influence CB₁ trafficking, perhaps through crosstalk with Rab5-mediated pathways via shared adaptor proteins. While Rab4 is widely known to reside in early endosomes and mediate rapid recycling (e.g. van der Sluijs et al., 1992b; Yudowski et al., 2009), it has also recently been suggested to control some aspects of early endosomal sorting (van der Sluijs et al., 1992b; Cormont et al., 1996; McCaffrey et al., 2001; Hall et al., 2004; Fernandez et al., 2009) and at least one adaptor protein, Rabaptin-5, interacts with both Rab5 and Rab4 in their GTP-bound conformations (Vitale et al., 1998). Over-expression of wild-type and Q67L Rab4 have also been reported to produce abnormalities in vesicular acidification and early endosomal structure (van der Sluijs et al., 1992b; McCaffrey et al., 2001), suggesting an additional mechanism via which CB₁ post-endocytic trafficking may have been mildly inhibited in this instance.

Perhaps the most surprising results presented in this chapter were those for Rab7. Rab7 is a mediator of early to late endosome transport and lysosome biogenesis (Feng et al., 1995; Press et al., 1998; Bucci et al., 2000; McCaffrey et al., 2001; Rink et al., 2005) and perhaps also proteasomal degradation (Mukherjee et al., 2005; Melnikov and Sagi-Eisenberg, 2009). Evidence presented in chapter four suggests that CB₁ undergoes a measurable degree of constitutive turnover within a matter of hours, while previous studies suggest CB₁ is degraded via both lysosomes and the proteasome system (Andersson et al., 2003; Rozenfeld and Devi, 2008). Indeed, of all the wild-type Rab proteins visualised, Rab7 appeared at low magnification to be the most closely colocalised with CB₁ in un-stimulated cells. However, on closer inspection by confocal microscopy, CB₁ and Rab7 were indeed regionally colocalised however CB₁-positive clusters seemed interspersed between Rab7-positive clusters. Although increased cytoplasmic CB₁ was observed with the dominant-positive mutant, contrary to
expectations the dominant-negative mutant did not appear to increase total CB₁, that is, prevent constitutive degradation. Meanwhile, a significant increase in basal surface CB₁ and a trend towards increased inverse-agonist induced surface upregulation were noted in cells expressing the dominant-negative mutant.

Before discussing the potential implications of the Rab7 dominant-negative mutant results, it is important to note that this Rab construct exhibited lower transfection efficiency (70.0 ± 6.2%, \( p = 0.024 \)) and overall lower apparent expression levels than its wild-type counterpart (as detected by the chimerised EGFP tag). As already discussed, the act of transient transfection seemed to reduce overall CB₁ expression. Although not thoroughly tested, it seems reasonable to hypothesise that the extent of introduced construct expression would correlate with overall CB₁, and perhaps other cellular protein, expression. If this is true, low Rab7-GDP construct expression may have allowed a greater rate of CB₁ synthesis in comparison with cells expressing wild-type Rab7 and thereby give the illusion of increased surface CB₁ via a mechanism not related to Rab7 function. However, as the general effect of transient transfection on CB₁ expression seems to influence surface and intracellular CB₁ equally, if the increase in surface expression noted with Rab7-GDP was a non-specific effect caused by a low expression level an analogous increase in cytoplasmic CB₁ would also be expected. The effect of the Rab7 dominant-negative mutant may therefore be real and not due to artefact. The reason for the lower apparent level of Rab7-GDP construct expression is not known. As already mentioned, DNA concentrations were verified multiple times and more than one DNA preparation was used over the course of the experiments rendering it unlikely that DNA quantity or quality was the culprit. Although the Rab protein coding region of the DNA constructs were sequence-verified, the EGFP and remainder of the vector were not. Perhaps a mutation in the CMV promoter resulted in a reduced level of protein synthesis from the construct, or a mutation in the EGFP sequence altered the fluorescent properties of the resulting protein, thereby providing an inaccurate representation of the Rab7 protein present. Unfortunately these
possibilities were not considered until the experiments in this chapter were concluded, however it would be advisable to investigate these prior to continuing research with this construct.

Therefore, assuming that the effects on CB₁ basal and SR-induced surface expression are genuine, the presence of the Rab7 dominant-negative mutant may have redirected a small amount of endocytosed or intracellular pool CB₁ to the cell surface. This scenario, rather than prevention of endocytosis, is more likely because the effect was also evident in the SR upregulation assay which is only sensitive to receptor delivery to the surface. The finding parallels that obtained with GASP-1, an adaptor protein involved in CB₁ degradation, whereby disruption of GASP-1 interactions appeared to promote CB₁ delivery to the surface (Martini et al., 2007; Tappe-Theodor et al., 2007), and again suggests that CB₁ has the potential to recycle under specific conditions. The lack of influence of Rab7-GDP on the recycling experiment indicates that the increased surface delivery was more likely due to mobilisation of the intracellular pool rather than bona fide recycling of internalised receptor, however this experiment measured recycling following agonist-induced internalisation, whereas constitutive internalisation could conceivably confer different trafficking potential. As the observed effect was small in magnitude, it would certainly be interesting to repeat this study having investigated the expression level issue noted above, and to compare the effects of this N125I mutant with the other commonly used dominant-negative construct, S22N, and/or following endogenous Rab7 knock-down by RNA interference.

The unexpected result of the Rab7 negative mutant’s lack of effect on overall CB₁ expression leads to the conclusion that CB₁ degradation is not Rab7-mediated. It should be noted that only basal degradation can be considered here, since agonist-induced degradation was not investigated in this study. Further, it would be preferable to confirm this result with the alternative methods already noted before fully discounting Rab7. A remote possibility for the lack of appreciable effects being artefactual is the use of the canine rather than human Rab7 sequence in a human host cell line. However, this seems unlikely as the sequences are only
one amino acid different and the canine construct has successfully inhibited degradation in human cell lines previously (Dube et al., 2009). Although Rab7 is the only Rab protein commonly identified to play a direct role in protein degradation, a number of the 60 or so Rab family members are as yet poorly characterised (Schwartz et al., 2008; Stenmark, 2009); CB₁ constitutive degradation may be mediated by one of these or alternatively not be Rab protein-dependent.

As the results in this chapter and commentary thus far demonstrate, the interpretation of results from Rab mutant transient expression is far from straightforward. It was certainly an advantage in this instance to measure surface and intracellular receptor expression separately, as reliance on ratiometry would have resulted in even greater ambiguity in the interpretation of results. Unfortunately, with exception of Rab5 and Rab7, the trends observed did not reach statistical significance. In these cases, although the null hypothesis (that no change occurred compared to the wild-type Rab) cannot be rejected, based on changes in CB₁ localisation evident upon visual inspection of the cells, the trends quantitated may be real effects that were not large enough or not consistent enough to reach significance under the experimental conditions used. Furthermore, the trending effects for Rab4 were consistent between the two isoforms tested. However, the actuality of these changes must remain conjecture until experiments with the necessary power to confirm these results statistically are performed. As already commented upon, transient transfection had a negative effect on cell health and also induced an overall reduction in CB₁ expression which may have contributed to increased variability, and therefore greater standard errors, between experiments. In addition, the extent of effects observed may have been slightly under-estimated in the quantification method used because all cells in the field of view are sampled, whether or not co-expressing the transiently-transfected protein. Although this is perhaps a minor drawback of the presently utilised quantification method, as the transfection protocol was optimised to produce a high efficiency of expression only a minor effect on the data was anticipated. The impact of the un-transfected cells may have been slightly greater than anticipated due to the effect of transient transfection in decreasing CB₁.
expression in transfected cells. To address these potential issues, a modified quantification approach was considered whereby the "Cell Scoring" MetaMorph® drop-in assay was used to identify the boundaries of EGFP/Rab-positive cells, and subsequent CB₁ analysis was limited to these boundaries thereby excluding EGFP/Rab-negative cells. Unfortunately this approach was found to be inappropriate because bright EGFP areas tended to result in disproportionately large cell areas due to out of focus blur. As previously mentioned, assaying for CB₁ effects at a later timepoint was considered and may have produced more pronounced Rab effects, however the viability of cells appeared to be compromised at later timepoints and cell health was considered to be of paramount importance in this study. Conversely, significant effects were obtained in response to Rab5 constructs so perhaps other trends noted were genuinely subtle effects as a result of indirect interactions with CB₁ and/or crosstalk between trafficking pathways.

The principle objective addressed in this chapter was to assess the effects of Rab GTPases on basal CB₁ localisation and compare the results with an influential prior study (Leterrier et al., 2004). While some caveats on the data have been discussed and an expansion of this study to address these would be required before the data can be considered definitive, the advantages of measuring surface and intracellular receptor expression separately with a high-throughput method as opposed to a ratiometric approach are obvious. Importantly, the balance of evidence garnered from Rab5 and Rab4 do not support the interpretation and conclusions of Leterrier et al. and instead further corroborate the conclusions of chapter four that CB₁ is not a recycling receptor and the intracellular pool does not form part of a constitutive endocytosis and recycling loop.
For the majority of receptor-mediated drug treatments, the effect on any given cell will largely be a consequence of the number of receptors at the cell surface. Considerable research in recent times has therefore focused on understanding the factors that regulate cell surface receptor expression, in particular, GPCR intracellular trafficking. While significant mechanistic insights into general cellular trafficking pathways have been gained, it is becoming increasingly clear that different receptor types undergo differential trafficking that is tightly regulated through interactions between receptor motifs and an array of adaptor and effector molecules (eg. Cao et al., 1999; Tanowitz and von Zastrow, 2003; Vargas and Von Zastrow, 2004). The investigation and discovery of such molecular mechanisms must be underpinned by a thorough understanding of the underlying trafficking characteristics of the receptor of interest. Indeed, some GPCRs, such as the β2-adrenergic receptor, are very thoroughly characterised and a number of advances have been made towards understanding the precise mechanisms controlling trafficking of these receptors. Relatively speaking, however, the study of CB₁ trafficking is in its infancy.

Both in vivo and in vitro, CB₁ is known to exhibit considerable cytoplasmic “intracellular pool” expression in addition to being present at the cell surface. The existing literature has established that CB₁ undergoes both constitutive and agonist-induced internalisation and is widely accepted to undergo proteolytic degradation in response to chronic agonist stimulation, a phenomenon linked to the development of tolerance to cannabinoid ligands in vivo. A few
studies have also provided evidence towards endocytic CB₁ undergoing recycling, and a hypothesis whereby CB₁ cycles between the surface and intracellular pool in a constitutive trafficking loop has gained acceptance in the cannabinoid field (e.g. D’Antona et al., 2006; Bari et al., 2008; Daigle et al., 2008a; Wu et al., 2008).

The exciting prospect at the outset of this study was therefore to further investigate CB₁ recycling with a view towards deciphering aspects of the mechanisms controlling trafficking via this pathway. Unfortunately, these originally intended aims were stalled when the results of pilot experiments suggested that CB₁ did not recycle. It therefore became clear that a thorough and in-depth characterisation CB₁ trafficking was called for, the results of which are detailed in chapters four and five. Overall these results suggest that CB₁ is a primarily degradative receptor that does not recycle following constitutive or agonist-induced internalisation. Concordantly, the data suggests that the intracellular pool is not formed by constitutive internalisation, nor acts as a receptor reservoir that may be mobilised to populate the cell surface. Therefore, while the originally intended aims of this project were to corroborate and extend the previously established CB₁ recycling hypothesis, the conclusions reached ultimately indicate the need for a revision of generally held theories and re-interpretation of prior studies.

**Method optimisation**

Techniques and methodologies are at the foundation of any scientific endeavour. It was recognised at the outset of this project that the proposed aims could only be achieved if the study was underpinned by a quantitative method that was appropriate for the subject matter, sensitive and reliable. The approach selected combined immunocytochemistry, which facilitated versatile receptor detection, and quantitative fluorescent image analysis with the Discovery-1™ high-throughput imaging platform and MetaMorph® processing software.

High-throughput imaging systems are powerful and versatile tools which are increasingly being utilised in both basic research and drug discovery (Giuliano et al., 2003). The automated
nature of the image acquisition and analysis used in this project confer significant advantages over labour-intensive methods such as radioligand assays and ratiometric image analysis. Importantly, these systems allow the analysis of thousands of cells under identical conditions with little risk of operator-introduced bias or judgement-based inconsistencies. The selected analysis algorithm produces a measure of the average total fluorescent signal arising per cell. This value is logical with regard to measuring protein expression levels and, when combined with selective immunodetection techniques, is applicable in the quantification of a range of biochemical assays. While a degree of human intervention is required to set analysis parameters, these are set based on objective judgements such as the approximate size of nuclei or the readily discernable boundary between receptor staining and background noise, and are applied equally across experimental treatments. Non-specific primary and secondary antibody staining on negative control cells was quantified as being marginally greater than 0% of positive control cell staining, indicating that the background thresholding method produced a good approximation of zero while maintaining sensitivity to very low level signals.

Over the course of this study there was one instance in which the sensitivity of the quantification method was not sufficient to detect a change that was discernable by visual inspection. This occurred when studying the pplss-hCB1 HEK cell line in which receptor expression was greatly exaggerated compared to the other cell lines studied. As Discovery-1™ is a widefield microscope, each image captures light that is both in and out of focus. For typical levels of staining produced by the non-pplss cell lines in this study, the out of focus light appears as a faint “blur” around edges of cells and is eliminated when a threshold is applied as for TGVC, yet low levels of staining remain detectable above the threshold level. However, for the pplss cell line the intensity of the out-of-focus blur exceeded that of the lowest true receptor staining and thereby when an appropriate threshold for the basal level of receptor staining was applied, some assay sensitivity for detecting low receptor expression was lost. Although not explored in this study, application of deconvolution algorithms to reduce the influence of out-of-focus blur may have alleviated some of this problem. In addition, a number of new high-
throughput imaging platform models are confocal microscopy-based and would therefore be much less vulnerable to artefact caused by unfocused light. This observation with the pplss cell line is, however, a cautionary reminder that it is important to monitor the phenomenon of interest as directly as possible and consider the raw data as well as the processed quantification in order to safeguard against drawing incorrect conclusions when unanticipated quantification artefacts arise. This highlights one advantage of imaging-based approaches, as opposed to other modalities such as ELISA and flow cytometry, in that the raw data can easily be evaluated qualitatively for comparison with quantitated results. Aside from the limitations noted when assessing the pplss cell line, the quantification approach selected and optimised for use in the present study represents a robust method that has proven useful for assessment of a wide range of trafficking processes in this study, and may well be applicable to other areas of hypothesis-based research and drug discovery.

Due to the extensive nature of the proposed project and range of experiments to be carried out, it was considered appropriate to utilise immortalised cell lines as opposed to primary cell lines which can be difficult or unethical to source in large numbers and present a number of technical challenges. All the cell types used (HEK, AtT-20, CHO, and Neuro-2a) possess amenable morphology for receptor localisation studies and widefield microscopy, and have been used in many previously published receptor trafficking and signalling studies (eg. see Wang et al., 2003; Liu-Chen, 2004; Tulipano and Schulz, 2007). Transfection of such cell lines with a receptor or protein of interest allows the researcher control over the protein construct design and level of expression. While transient transfection has been used in the past for some receptor studies, stable expression (where the introduced DNA construct is incorporated into the host cell genomic DNA) was preferred in this study as the method supports consistent receptor expression over time and is less likely to negatively influence cell health. rCB1 expression levels in the HEK cell line selected were previously quantified to be equivalent to that found in a number of brain regions (Kearn et al., 2005; Glass et al., 1997), while hCB1 was expressed at lower levels in the CHO and AtT-20 cells. It is therefore unlikely that the results in
this study could have been influenced by artefact due to receptor over-expression. The transfected receptor constructs were chimerised with an HA tag at the receptor amino-terminus to facilitate receptor detection. Inclusion of the epitope tag in the extracellular domain of the receptors allowed the discrimination of surface from total receptors simply by performing immunolabelling under conditions that do not permeabilise the cell membrane. Such selective antibody labelling holds an advantage over the use of fluorescent tags (such as GFP), where delineation of cell compartments must be performed visually through observation of microscope images, or by colocalisation with additional compartment-specific markers. Further, as GFP is approximately 50% of the molecular weight of most GPCRs (27 kDa) it is not surprising that the chimerisation of GFP has been noted to markedly alter the trafficking behaviour of GPCRs (McLean and Milligan, 2000). In contrast, the 10 amino acid HA epitope contributes to less than 3% of the molecular weight when expressed on most GPCRs. Although the introduced receptor constructs were localised similarly to \textit{in vivo} CB\textsubscript{1} and produced signalling responses as expected, it was considered important to compare the results from transfected cells with a cell line expressing CB\textsubscript{1} endogenously. Such a model would not be subject to artefact potentially produced by transfection or inclusion of the HA tag. Key experiments were therefore also carried out with the Neuro-2a cell line which is of neuronal lineage and does indeed express CB\textsubscript{1} endogenously.

Immunocytochemistry techniques formed the basis for the majority of findings in this report. As HA-tagged receptors were studied in most of the assays, a commercially available anti-HA antibody which was shown to be effective and specific for detection of the HA epitope could be utilised. However as discussed above, it was also intended that untagged CB\textsubscript{1} be studied. Unfortunately, attempts to validate an array of commercially available antibodies directed against the CB\textsubscript{1} amino terminus revealed low efficacy and, in a number of cases, poor specificity for detecting CB\textsubscript{1}. Although the work presented here relates specifically to CB\textsubscript{1} antibodies, the literature suggests these issues also plague many other fields using immunodetection methods (eg. Pozner-Moulis et al., 2007; Couchman, 2009). This state of
affairs, with the onus seemingly on researchers to verify the authenticity of commercially available products, is somewhat disturbing. For the most part, labs utilising immunodetection techniques invest substantial funds to purchase antibodies, yet may also be required to undertake costly antibody validation studies to confirm manufacturer claims, or in the case that the antibody is not performing as expected, to produce evidence that will warrant refund of the purchase price. It seems that in the interest of consumer rights, the antibody manufacturer should take responsibility to ensure the product is suitable for the intended use prior to marketing it as such. Commercial producers have traditionally used ELISA-based methods to verify antibodies, however the detection of a pure antigenic peptide by ELISA is not necessarily a good measure of how the same antibody will behave in a complex protein mixture where affinity then becomes important (Rhodes and Trimmer, 2006). With ever-advancing improvements in tissue array and high-throughput screening technologies, large numbers of antibody clones can now be screened quickly. These types of methods facilitate testing under conditions that are much closer to the applications for which they will ultimately be used by researchers, and it is hoped that more antibody manufacturers will adopt these quality control measures to complement ELISA-based approaches in the near future. At the same time, perhaps researchers should keep in mind that antibody production is a complex process reliant on biological processes. Selecting a reliable antibody manufacturer and keeping lines of communication open – both when things go wrong and when an antibody is working as expected – can help both the researcher and manufacturer to solve problems quickly and maintain high production standards (Kalyuzhny, 2009). Additionally, multiple web-based initiatives for collaborative validation and reporting of antibodies have been founded recently (eg. Major et al., 2006; Bjorling and Uhlen, 2008) and some commercial websites also allow end-users to share antibody reviews and protocols for their use (eg. Abcam, http://www.abcam.com). With input from researchers, these types of resources may ultimately provide a catalogue of validated antibodies, reducing the need for extensive antibody characterization by individual groups before research is undertaken. Fortunately, the generous
donation of privately-generated anti-CB₁ antibodies from a colleague ultimately allowed endogenous CB₁ to be studied as part of this project.

**CB₁ is a non-recycling receptor**

Having demonstrated that CB₁ underwent agonist-induced internalisation in a similar manner to that previously observed, the drug conditions under which CB₁ recycling might be observed were investigated by monitoring net surface repopulation. CB₁ did indeed repopulate the plasma membrane, however receptors would only accumulate at the surface in the presence of a sufficiently competitive concentration of inverse-agonist, indicating that the cannabinoid agonists could not be washed out of the assay system. This is an important finding for consideration when researching cannabinoid drug responses and particularly in the interpretation of assays that call for agonist washout such as are commonly applied in studying desensitisation.

As the observation of net repopulation of the cell surface could be indicative of recycling or delivery of newly synthesised receptors, the question of whether CB₁ undergoes *bona fide* recycling was approached from a number of angles. A quintessential assay for receptor recycling whereby antibody-tagged receptors are monitored did not detect any CB₁ recycling, yet in parallel studies D₁ rapidly recycled demonstrating the appropriateness of this assay. As well as this non-recycling result being replicated in three other cell systems, internalised CB₁ was localised very differently to D₁, appearing diffusely cytoplasmic whereas D₁ was contained in a perinuclear area. CB₁ also underwent degradation instead of remaining sequestered in the cytoplasm, suggesting that the potential for recycling was not retained. Although it has previously been suggested that long or high-concentration agonist stimulations might divert CB₁ to a degradative pathway (Hsieh et al., 1999; Martini et al., 2007), it is unlikely either of these mechanisms is at play here because a short agonist stimulation and two different agonists at the lowest concentrations that consistently produced near-complete internalisation were used.
CB₁ was also observed to undergo constitutive internalisation. As this process has been suggested to form part of a constitutive recycling loop (Leterrier et al., 2004), detection of recycling following an extended period of live antibody feeding was also attempted. Again no evidence of recycling was detected in the rCB₁ HEK cell line. However, a very small extent of recycling, estimated to account for less than 1% of the basal surface CB₁ expression level, was observed for the pplss hCB₁ HEK cell line. Due to the gross over-expression of CB₁ in this cell model it seems likely that this small amount of recycling resulted from saturation of the degradation pathway CB₁ would normally traverse rather than a physiologically relevant effect. This mechanism might also explain the recycling results presented in Martini et al. (2007) whose receptor expression construct also included an N-terminal signal sequence.

It therefore seems that the repopulation of surface receptor following agonist-induced internalisation is due to delivery of newly synthesised receptor and indeed, a protein synthesis inhibitor blocked surface repopulation. This observation was in contrast to the findings of two previous studies in which similar assays were performed (Hsieh et al., 1999; Tappe-Theodor et al., 2007), however the exact conditions under which the protein synthesis inhibitors were applied might have influenced the results; in this study particular care was taken to select the concentration of cycloheximide applied to cells to increase the likelihood cell health and normal functioning would be maintained. Furthermore, both previous studies were reliant on qualitative observations from confocal images and some data interpretation may have been misguided. For instance, in one study (Tappe-Theodor et al., 2007) the disappearance of intracellular puncta was taken to indicate recycling had occurred, yet the surface expression observed following “recycling” appeared very similar to that remaining at the end of the internalisation period which could equally be taken to indicate that internalised CB₁ had degraded.

Finally, neither Rab4 nor Rab11, commonly cited recycling pathway mediators, influenced basal surface CB₁ expression nor altered the outcome of the antibody live-feeding recycling experiment. The Rab4 result is in contrast to the findings of Leterrier et al. (2004). Potential
reasons for this discrepancy are discussed in detail in chapter five (pg. 148), however the quantification methods employed in the present study appear superior to those used in the 2004 study, and on balance this result provides further evidence against the hypothesis that CB₁ recycles.

While the results of the present study are consistent between four cell models, one of which expresses CB₁ 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 CB₁ trafficking. One quality of CB₁ trafficking in hippocampal primary culture neurons that has now been reported by two groups, but that was not replicated in the current study, was that of constitutively internalised CB₁ being trafficked (and therefore recycled) to the axonal plasma membrane (Leterrier et al., 2006; McDonald et al., 2007). 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 utilised chimerisation of a fluorescent tag and transient receptor construct expression; two techniques with reasonable potential to artefactually influence trafficking pathways. In addition, it should be noted that studies on cultured primary neurons are not without limitations; these cells exhibit considerable CB₁ 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 (eg. Katona et al., 1999; Bodor et al., 2005; Matyas et al., 2006). CB₁ is also expressed on some non-polar cell types such as microglia (Waksman et al., 1999) and peripheral immune cells (Galiègue et al., 1995), which the presently studied cell lines may well model very closely. It would certainly be of interest, however, to continue detailed characterisation studies in model systems that replicate in vivo conditions as closely as possible. An approach with the potential to be particularly illuminating in the study of CB₁ trafficking was utilised by Csaba et al. (2007) to study somatostatin type 2 receptor trafficking. Here, animals were subjected to acute drug
treatments and the effects on hippocampal receptor localisation were assessed by immunohistochemistry. Demarked laminar CB$_1$ expression would allow clear delineation of changes in CB$_1$ localisation, and the approach could perhaps also be adapted to hippocampal slice culture which would allow more directed control of drug treatments and receptor detection techniques while maintaining neuronal cells in their original architectural context.

**Source and role of the CB$_1$, intracellular pool**

In addition to the apparent non-recycling CB$_1$, phenotype, a second finding of the present study that is in opposition to currently held theories is that the CB$_1$ intracellular pool represents receptor that is neither formed by internalised receptors, nor is constitutively mobilised to the cell surface, and therefore does not contribute to CB$_1$ signalling via classical paradigms. Again, the evidence for this conclusion comes from multiple experimental approaches. An important feature of the present work, and unique in comparison to recent studies addressing similar questions, is the application of a proteolytic method to directly quantitate intracellular receptors.

The study was initiated with a simple immunocytochemical observation of cellular receptor distribution. In agreement with general observations in the field, a sizeable proportion of CB$_1$ was expressed intracellularly under basal conditions. Introduction of a signal sequence (pplss) designed to increase the efficiency of N-terminal translocation in the ER did indeed markedly increase the net expression levels of the receptor as previously reported (Andersson et al., 2003), however did not appear to influence formation of the intracellular pool suggesting that the receptors are not retained intracellularly due to poor ER translocation. Furthermore, western blot analysis indicated that, for at least two of the three cell lines analysed, the majority of intracellular receptor species were full-length and glycosylated as for surface CB$_1$.

Despite the majority of intracellular CB$_1$ appearing to be fully-formed and processed, blockade of constitutive internalisation with inverse-agonist produced no change in the size of the intracellular pool, whereas the constitutively-cycling theory would anticipate a reduction in the
in intracellular pool due to mobilisation of receptors to the surface. Further, antibody labelled CB\(_1\) was observed to degrade following constitutive internalisation rather than become sequestered in the intracellular pool. Although a recent report suggested that intracellular CB\(_1\) is the predominant signalling population (Rozenfeld and Devi, 2008), this appears unlikely based on the present data as the intracellular pool did not colocalise with G-alpha proteins known to mediate CB\(_1\) signalling, nor were intracellular receptors degraded in response to chronic agonist stimulation. Finally, blockade of CB\(_1\) constitutive internalisation with a dominant-negative Rab5 mutant induced surface upregulation, however did not alter the size of the intracellular pool. Interestingly, these findings conflict with conclusions made by Leterrier et al. (2004) in a similar study, however when data was analysed with an analogous calculation the results were comparative. This indicates that although the underlying data for this Rab isoform may have been very similar between the two studies, a reliance on indirect measures may have lead to incorrect inferences being drawn, again demonstrating that direct quantitation of receptor expression in different cellular compartments is superior to the ratiometric approach.

**Rab GTPases and CB\(_1\) trafficking**

Of the Rab GTPases studied, the results with Rab4, 5 and 11 have already been discussed in detail. The results from the final Rab protein studied, Rab7, were particularly surprising as contrary to expectations, introduction of the mutant forms did not appear to interfere with CB\(_1\) degradation. While some caveats on the data and its interpretation were discussed in detail in chapter four (pg. 148), it seems that CB\(_1\) trafficking is not directly mediated by Rab4, 7 or 11.

This brings to light an inherent difficulty when investigating a large family of related proteins, in that when it is not possible to study all or most of the family members a subset must be selected to investigate. While in this instance a primary objective was to perform a direct comparison with a previous study which therefore directed the selection of subtypes for this project, researchers are generally required to place an educated guess as to which Rab subtypes are likely to be important for their protein of interest. Although the well-characterised
Rab GTPases are fairly easily assigned depending on the trafficking pathway expected to be relevant, understandably Rab proteins with poorly understood function are unlikely to be selected for studies such as the one at present. On the other hand, if study of the seemingly already well-characterised Rab proteins is biased towards certain contexts or with particular trafficking pathways in mind, the detection of other roles for these family members may be hindered. It will be interesting to observe whether high-throughput methods such as that used here will be taken up by the field as an unbiased approach with the potential to screen the effects of large numbers of Rab GTPases on large numbers of cargo proteins. Another technique with increasing importance in the study of Rab GTPases is RNA interference (Simpson and Jones, 2005), whereby endogenous gene expression is silenced by the transient introduction of short RNA sequences complementary to mRNA (reviewed in Leung and Whittaker, 2005). This technique is already in use as a companion to dominant-negative mutant studies (eg. Krishnan et al., 2007; Zhang et al., 2008) and, assuming suitable RNA probes are available, this technique would also be conducive to high-throughput screening for Rab-mediated effects (Echeverri and Perrimon, 2006).

**Perspectives and future directions**

CB₁ is one of the most abundant GPCRs in the brain and a significant proportion of that receptor population resides in the cytoplasm (Pettit et al., 1998; Tsou et al., 1998; Katona et al., 1999). While it was previously inferred that this intracellular pool represented functional receptors with the potential for mobilisation to the cell surface and contribution to cannabinoid responsiveness (Leterrier et al., 2004), the data presented in this study suggests that these receptors are more likely to be non-functional and cannot be mobilized under normal circumstances. This also implies that aside from its important roles in brain function, high levels of CB₁ in the nervous system may partly be as a result of compensatory mechanisms for the significant degree of inefficiency in CB₁ 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. It appears that the majority of
intracellular pool CB1 is fully glycosylated and is not particularly influenced by signal sequence-enhanced ER translocation, however neither of these observations can confirm for sure whether or not intracellular pool receptors are correctly folded and completely processed. Whether or not intracellular pool CB1 is mature, it would certainly be interesting to pursue the mechanisms via which the intracellular pool is generated and investigate whether these pathways could be modulated to enhance correct processing and/or delivery of CB1 to the cell surface.

Post-translational modifications other than glycosylation which might differentiate intracellular from surface CB1 include phosphorylation, ubiquitination and palmitoylation; all of which have been correlated with differential targeting or fate of GPCRs previously. For example, surface delivery of adenosine A2a receptors can be enhanced by stimulating de-ubiquitination of intracellular receptors (Milojevic et al., 2006).

Whether or not modulated by post-translational modifications, active sorting of receptors between trafficking pathways is controlled by interactions with adaptor and effector proteins. Although the Rab proteins and their mutants, a small subset of which were studied in this project, can be useful as exploratory tools to assist in defining receptor trafficking pathways, the interpretation of data can be difficult and as discussed above, this approach can represent somewhat of an exercise in guessing the “right” Rab effector for the receptor and trafficking pathway of interest. Further, it is unlikely that general cellular trafficking adaptors represent viable targets for therapeutic intervention because of the potential for off-target effects on other receptor systems. However, a number of more directed approaches may be useful in identifying mechanisms that specifically control CB1 trafficking.

Direct interactions between receptors and effector or adaptor proteins generally occur via recognition sites referred to as motifs that consist of particular amino acid sequences in the appropriate spatial conformation and sub-cellular context. A number of both generalised and
receptor-specific motifs have been identified and these appear to play roles across a wide range of intracellular pathways. As well as recycling and degradation, signal motifs related to ER and Golgi processing, and post-Golgi targeting to intracellular organelles have been reported (reviewed in Dong et al., 2007) which may play a role in CB₁ trafficking to the intracellular pool. An interesting experiment would be to generate a set of receptor chimeras in which the cytoplasmic tail, or perhaps other non-membrane-spanning region, of CB₁ is attached to the end of, or as a replacement for, the equivalent region on another receptor with a different trafficking phenotype, and vice versa. Such an investigation could reveal whether the intracellular pool phenotype is dominant over the trafficking motifs present on the comparative receptor and assist in identifying the location of relevant CB₁ sequence regions. Further mutagenesis studies, including truncation, deletion or point mutation of regions of interest could assist in further refining the putative trafficking motif. Once motifs or wider regions of interest have been identified, library screening methods such as the 2-hybrid approaches might assist in identifying the cellular protein interacting with the receptor to produce a functional response. At least one CB₁ interacting protein has already been identified via this approach (Niehaus et al., 2007).

There is also potential for direct ligand-mediated modification of receptor trafficking in that pharmacological chaperones have also been described that can increase the efficiency of GPCR folding and maturation and/or stability following synthesis (Petäjä-Repo and Bouvier, 2005). This type of mechanism might also be applicable to CB₁, particularly as the majority of cannabinoid ligands are lipophilic and therefore have the inherent ability to cross cell membranes and potentially interact with intracellular sites. In addition, allosteric modulators have recently been described for CB₁ (Price et al., 2005). These may confer unique effects on receptor conformation and it would certainly be interesting to investigate whether these exert any influence on CB₁ trafficking.
Once identified via the above-mentioned approaches, the mechanisms through which trafficking of CB\textsubscript{1} to or from the intracellular pool could be exploited to enable completed processing and/or re-routing of these receptors to the plasma membrane. Conversely, such investigations may reveal mechanisms via which endocytosed CB\textsubscript{1} could be recycled rather than degraded. As receptor trafficking processes control the number of receptors at the cell surface and therefore are integral to ligand responsiveness, knowledge of the molecular mechanisms controlling trafficking could potentially be exploited therapeutically to alter receptor localisation and thereby modulate endogenous or exogenous ligand effects, or alter the development of ligand tolerance. Indeed, a GASP-1 (mediator of CB\textsubscript{1} degradation) knockout mouse model exhibits reduced tolerance to WIN (Martini et al., 2010), which may occur via redirection of CB\textsubscript{1} trafficking from degradation to recycling (Martini et al., 2007; Tappe-Theodor et al., 2007).

In addition, a number of reports suggest that dysregulation of receptor trafficking can be pathogenic (eg. Szebenyi et al., 2003; reviewed in Conn et al., 2007). Although CB\textsubscript{1} trafficking dysfunction has yet to be specifically implicated in disease aetiology, this is certainly a feasible mechanism via which aberration of CB\textsubscript{1} function might occur. An exciting preliminary finding from the laboratory where the present research was carried out suggests that the preferential loss of CB\textsubscript{1} compared with other colocalised receptors observed in human Huntington’s disease can be recapitulated in a model cell line and appears to be the result of differential dysregulation of trafficking pathways (E Scotter and M Glass, unpublished observation). The identification and characterisation of CB\textsubscript{1}-specific trafficking pathway interactors and modulators may therefore represent exciting prospects for the development of therapeutics directed towards the endocannabinoid system.

In conclusion, the research described in this thesis has demonstrated the development of a quantitative method suitable for high-throughput assessment of receptor trafficking, determined that CB\textsubscript{1} does not recycle following agonist-induced or constitutive internalisation and is instead
degraded, and provided evidence to suggest that the widely-noted intracellular pool represents a receptor reservoir not delivered to the cell surface and awaiting degradation. The insight gained in this thesis will certainly serve as an important foundation for further investigations into the molecular mechanisms that control sorting of CB₁ to different pathways. The potential roles in modulating the actions of cannabinoid therapeutics, and perhaps disease processes, reinforce the importance of continued rigorous research into CB₁ trafficking.
## Appendices

### Details of statistical tests

Notes to Table 7.1 are provided below (pg. 174).

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<td>Paired $t$-test</td>
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<td>(NA)</td>
<td>$t(2) = -4.69$</td>
<td>0.043</td>
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<td>Pass, $p = 0.58$</td>
<td>$F$(labelling: 1.27) = 0.25</td>
<td>Labelling: 0.62</td>
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<td>1-way RM ANOVA</td>
<td>Pass, $p = 0.13$</td>
<td>Pass, $p = 0.84$</td>
<td>$F(3,6) = 279.31$</td>
<td>&lt;0.0001</td>
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<td>Pass, $p = 0.60$</td>
<td>Pass, $p = 0.97$</td>
<td>$F(3,6) = 3.91$</td>
<td>0.073</td>
<td>(NA)</td>
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<td>1-way RM ANOVA</td>
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<td>Pass, $p = 0.27$</td>
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<td>&lt;0.0001</td>
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<td>Pass, $p = 0.34$</td>
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<td>&lt;0.001</td>
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<td>Pass, $p = 0.62$</td>
<td>Pass, $p = 0.59$</td>
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<td>Pass, $p = 0.10$</td>
<td>$F$(labelling: 2.6) = 0.043</td>
<td>Labelling: 0.85</td>
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<td>Pass, $p = 0.22$</td>
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<td>Pass, $p = 0.43$</td>
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<td>$t(3) = 2.25$</td>
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<td>Pass, $p = 0.39$</td>
<td>$F(4) = 0.29$</td>
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<td>SR-induced upregulation vs repopulation rates</td>
<td>t-test</td>
<td>Pass, $p = 0.54$</td>
<td>Pass, $p = 0.87$</td>
<td>$F(4) = -0.39$</td>
<td>0.72</td>
<td>(NA)</td>
</tr>
<tr>
<td>4 / 112</td>
<td>Constitutive internalisation vs CHX-induced surface downregulation t½s</td>
<td>t-test</td>
<td>Pass, $p = 0.42$</td>
<td>Pass, $p = 0.57$</td>
<td>$F(4) = -0.72$</td>
<td>0.51</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 123</td>
<td>Transfection methods, EGFP-positive cells</td>
<td>1-way ANOVA</td>
<td>Pass, $p = 0.12$</td>
<td>Pass, $p = 0.92$</td>
<td>$F(2,11) = 14.7$</td>
<td>0.0008</td>
<td>Tukey, 7.11</td>
</tr>
<tr>
<td>5 / 123</td>
<td>Transfection methods, cell counts (compared to noDNA)</td>
<td>KW 1-way ANOVA on Ranks</td>
<td>(NA)</td>
<td>$H(3) = 11.62$</td>
<td>0.0088</td>
<td>Dunn's vs control, 7.12</td>
<td></td>
</tr>
<tr>
<td>5 / 125</td>
<td>Basal surface CB₁, wt Rabs vs GFP (excluding noDNA)</td>
<td>1-way RM ANOVA</td>
<td>Pass, $p = 0.24$</td>
<td>Pass, $p = 0.28$</td>
<td>$F(5,15) = 1.86$</td>
<td>0.16</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 125</td>
<td>Basal total CB₁, wt Rabs vs GFP (excluding noDNA)</td>
<td>1-way RM ANOVA</td>
<td>Pass, $p = 0.32$</td>
<td>Pass, $p = 0.57$</td>
<td>$F(5,15) = 0.48$</td>
<td>0.79</td>
<td>(NA)</td>
</tr>
</tbody>
</table>

continued next page
<table>
<thead>
<tr>
<th>Chapter / page reported</th>
<th>Description of data under comparison</th>
<th>Statistical test</th>
<th>Normality test result</th>
<th>Equal variance test result</th>
<th>Statistical test result</th>
<th>p-value</th>
<th>Post-test result, table</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 / 125</td>
<td>Basal surface CB1, noDNA vs GFP (100%)</td>
<td>One sample t-test to a hypothetical value</td>
<td>Pass, ( p = 0.70 )</td>
<td>(NA)</td>
<td>( F(3) = 3.18 )</td>
<td>0.050</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 125</td>
<td>Basal total CB1, noDNA vs GFP (100%)</td>
<td>One sample t-test to a hypothetical value</td>
<td>Pass, ( p = 0.50 )</td>
<td>(NA)</td>
<td>( F(3) = 9.70 )</td>
<td>0.0023</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 128-129</td>
<td>Basal CB1, Rab5 mutants vs wt</td>
<td>2-way RM ANOVA</td>
<td>Fail, ( p &lt; 0.05 )</td>
<td>Fail, ( p &lt; 0.05 )</td>
<td>( F(Staining \times \text{Mutant}: 6,6) = 13.49 )</td>
<td>Staining x Mutant: 0.0030</td>
<td>Tukey, 7.13</td>
</tr>
<tr>
<td>5 / 132</td>
<td>Basal CB1, Rab4a mutants vs wt</td>
<td>2-way RM ANOVA</td>
<td>Pass, ( p = 0.31 )</td>
<td>Pass, ( p = 1 )</td>
<td>( F(Staining \times \text{Mutant}: 6,6) = 0.71 )</td>
<td>Staining x Mutant: 0.87</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 132</td>
<td>Basal CB1, Rab4b mutants vs wt</td>
<td>2-way RM ANOVA</td>
<td>Fail, ( p &lt; 0.05 )</td>
<td>Fail, ( p &lt; 0.05 )</td>
<td>( F(Staining \times \text{Mutant}: 6,6) = 0.87 )</td>
<td>Staining x Mutant: 0.87</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 132</td>
<td>Basal CB1, Rab11 mutants vs wt</td>
<td>2-way RM ANOVA</td>
<td>Fail, ( p &lt; 0.05 )</td>
<td>Fail, ( p &lt; 0.05 )</td>
<td>( F(Staining \times \text{Mutant}: 6,6) = 0.21 )</td>
<td>Staining x Mutant: 0.21</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 139</td>
<td>Basal CB1, Rab7 mutants vs wt</td>
<td>2-way RM ANOVA</td>
<td>Fail, ( p &lt; 0.05 )</td>
<td>Fail, ( p &lt; 0.05 )</td>
<td>( F(Staining \times \text{Mutant}: 6,6) = 5.96 )</td>
<td>Staining x Mutant: 0.020</td>
<td>Tukey, 7.14</td>
</tr>
<tr>
<td>5 / 142</td>
<td>SR upregulation, mutant vs wt Rabs</td>
<td>1-way RM ANOVA</td>
<td>Pass, ( p = 0.19 )</td>
<td>Pass, ( p = 0.12 )</td>
<td>( F(8,22) = 2.3 )</td>
<td>0.059</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 144</td>
<td>Cell count 48 h post-transfection, Rab11 dominant-negative vs wt (100%)</td>
<td>One sample t-test to a hypothetical value</td>
<td>Pass, ( p = 0.39 )</td>
<td>(NA)</td>
<td>( t(3) = 3.95 )</td>
<td>0.029</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 144</td>
<td>Cell count 18 h post-transfection, Rab11 dominant-negative vs wt (100%)</td>
<td>One sample t-test to a hypothetical value</td>
<td>Pass, ( p = 0.65 )</td>
<td>(NA)</td>
<td>( t(2) = 0.84 )</td>
<td>0.49</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 145</td>
<td>Transfection efficiency, all Rabs (excluding Rab7 dominant-negative)</td>
<td>1-way RM ANOVA</td>
<td>Pass, ( p = 0.17 )</td>
<td>Pass, ( p = 0.20 )</td>
<td>( F(8,21) = 0.74 )</td>
<td>0.65</td>
<td>(NA)</td>
</tr>
<tr>
<td>5 / 151</td>
<td>Transfection efficiency, Rab7 dominant-negative vs wt (100%)</td>
<td>One sample t-test to a hypothetical value</td>
<td>Pass, ( p = 0.16 )</td>
<td>(NA)</td>
<td>( F(3) = 4.22 )</td>
<td>0.024</td>
<td>(NA)</td>
</tr>
</tbody>
</table>

Table 7.1 Statistical tests performed to assess data
Notes to table of statistical tests (Table 7.1)

Advice on the appropriate statistical tests to perform was provided by Mr Stephen Vander Hoorn (Biostatistics Manager, Clinical Trials Research Unit, University of Auckland) and Dr Marion Blumstein (Senior Tutor, Student Learning Centre, University of Auckland). ‘RM’ or ‘paired’ refers to repeated measures or paired design. These designs were applied when the conditions for comparison were assessed within one experiment that was repeated multiple times. As the datapoints from each experiment were not independent of each other, each experiment was treated as one “subject”. The samples consisted of data from at least three independent experiments. Non-RM or paired designs were utilised when all data under comparison was obtained independently. (“KW”, Krusal-Wallis.)

Tests for normality of data and equality of variance were carried out where applicable. The tests were the Kolmogorov-Smirnov test for normality and Levene’s test for equal variance. A pass result was obtained if the \( p \) value for the test was greater than 0.05. When the assumptions of normality and equal variance were not met, non-parametric tests were used if possible. The statistics software packages utilised did not have the capability to perform non-parametric 2-way RM ANOVA tests and therefore some datasets that did not meet the normality and variance assumptions were analysed by the standard parametric method. Although this is not ideal, ANOVA is known to be very robust to non-normality and reasonably robust to heteroscedasticity (inequality of variance) (eg. Box and Andersen, 1955).

The “Test result” lists the test parameter, degrees of freedom (for ANOVA, \( df \)-between and \( df \)-within), and result. For ANOVA, post-hoc tests to assess differences between individual group means were only evaluated when there was found to be an overall difference within the group.

Results of ANOVA post-hoc multiple comparison tests

The results of Tukey (or Dunn’s for non-parametric analysis) post-hoc multiple comparisons tests following ANOVA are provided below (Table 7.2 - Table 7.14). “Factor” is the dataset
under comparison (for 2-way ANOVA); “Comparison” is a brief description of the datapoints under comparison; “Diff of Means” is the difference between the means in units relevant to the experiment; “Diff of Ranks” is the difference between the mean rank values (used in Dunn’s non-parametric test); “p” is the number of conditions/treatments under comparison; “q” is the test statistic; “P” is the p-value; “P<0.050” annotates whether the result is considered significant at an alpha level of 0.05. A result of “Do Not Test” occurs when the means under comparison are enclosed by two other means already found not to be significantly different to each other and should be treated as though no significant difference exists.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (no trypsin) vs SFM</td>
<td>99.7176</td>
<td>4</td>
<td>33.8643</td>
<td>0.0002</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle (no trypsin) vs FSM</td>
<td>2.5582</td>
<td>4</td>
<td>0.8688</td>
<td>0.9239</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>Vehicle (no trypsin) vs TIS</td>
<td>7.5658</td>
<td>4</td>
<td>2.5694</td>
<td>0.3512</td>
<td>Do Not Test</td>
</tr>
</tbody>
</table>

Plus 3 additional comparisons not pertinent to the investigation.

Table 7.2  Tukey post-test result for trypsin inhibitor optimisation, surface CB₁

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle vs. -6</td>
<td>55.9072</td>
<td>7</td>
<td>14.3193</td>
<td>0.0002</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -7</td>
<td>61.9028</td>
<td>7</td>
<td>18.0194</td>
<td>0.0002</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -8</td>
<td>39.478</td>
<td>7</td>
<td>11.4917</td>
<td>0.0002</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -9</td>
<td>8.0032</td>
<td>7</td>
<td>2.3297</td>
<td>0.6592</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>Vehicle vs. -10</td>
<td>4.3163</td>
<td>7</td>
<td>1.2564</td>
<td>0.9671</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>Vehicle vs. -11</td>
<td>4.5206</td>
<td>7</td>
<td>1.3159</td>
<td>0.9593</td>
<td>Do Not Test</td>
</tr>
</tbody>
</table>

Plus 15 additional comparisons not pertinent to the investigation.

Table 7.3  Tukey post-test result for monensin concentration response, surface CB₁
### APPENDICES

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle vs. -6</td>
<td>28.8674</td>
<td>7</td>
<td>5.9888</td>
<td>0.0148</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -7</td>
<td>22.2975</td>
<td>7</td>
<td>4.6258</td>
<td>0.0729</td>
<td>No</td>
</tr>
<tr>
<td>Vehicle vs. -8</td>
<td>15.5218</td>
<td>7</td>
<td>3.2201</td>
<td>0.3271</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>Vehicle vs. -9</td>
<td>4.4453</td>
<td>7</td>
<td>0.9222</td>
<td>0.9931</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>Vehicle vs. -10</td>
<td>0.3773</td>
<td>7</td>
<td>0.07827</td>
<td>1</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>Vehicle vs. -11</td>
<td>1.149</td>
<td>7</td>
<td>0.2384</td>
<td>1</td>
<td>Do Not Test</td>
</tr>
</tbody>
</table>

Plus 15 additional comparisons not pertinent to the investigation.

**Table 7.4** Tukey post-test result for monensin concentration response, cell counts

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle vs. -1</td>
<td>89.1192</td>
<td>7</td>
<td>19.4130</td>
<td>0.0002</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -2</td>
<td>54.2395</td>
<td>7</td>
<td>13.4280</td>
<td>0.0002</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -3</td>
<td>24.1226</td>
<td>7</td>
<td>5.9720</td>
<td>0.0175</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -4</td>
<td>8.6470</td>
<td>7</td>
<td>2.1407</td>
<td>0.7328</td>
<td>No</td>
</tr>
<tr>
<td>Vehicle vs. -5</td>
<td>5.9140</td>
<td>7</td>
<td>1.4641</td>
<td>0.9346</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>Vehicle vs. -6</td>
<td>7.9791</td>
<td>7</td>
<td>1.9754</td>
<td>0.7933</td>
<td>Do Not Test</td>
</tr>
</tbody>
</table>

Plus 15 additional comparisons not pertinent to the investigation.

**Table 7.5** Tukey post-test result for CHX concentration response, total CB1

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle vs. -1</td>
<td>24.7029</td>
<td>7</td>
<td>8.8711</td>
<td>0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -4</td>
<td>19.4835</td>
<td>7</td>
<td>7.9519</td>
<td>0.0022</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -3</td>
<td>16.8712</td>
<td>7</td>
<td>6.8857</td>
<td>0.0065</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -2</td>
<td>14.668</td>
<td>7</td>
<td>5.9865</td>
<td>0.0172</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle vs. -5</td>
<td>2.7124</td>
<td>7</td>
<td>1.107</td>
<td>0.9821</td>
<td>No</td>
</tr>
<tr>
<td>Vehicle vs. -6</td>
<td>1.4634</td>
<td>7</td>
<td>0.5973</td>
<td>0.9994</td>
<td>Do Not Test</td>
</tr>
</tbody>
</table>

Plus 15 additional comparisons not pertinent to the investigation.

**Table 7.6** Tukey post-test result for CHX concentration response, cell counts
### Table 7.7  Tukey post-test result for ConA concentration response, surface CB$_1$

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>-6 vs. Vehicle</td>
<td>51.4958</td>
<td>6</td>
<td>8.0844</td>
<td>0.0006</td>
<td>Yes</td>
</tr>
<tr>
<td>-7 vs. Vehicle</td>
<td>45.4967</td>
<td>6</td>
<td>7.1426</td>
<td>0.0017</td>
<td>Yes</td>
</tr>
<tr>
<td>-8 vs. Vehicle</td>
<td>35.4819</td>
<td>6</td>
<td>5.5704</td>
<td>0.0137</td>
<td>Yes</td>
</tr>
<tr>
<td>-9 vs. Vehicle</td>
<td>19.4154</td>
<td>6</td>
<td>3.0481</td>
<td>0.3126</td>
<td>No</td>
</tr>
<tr>
<td>-11 vs. Vehicle</td>
<td>15.6769</td>
<td>6</td>
<td>2.4611</td>
<td>0.5284</td>
<td>Do Not Test</td>
</tr>
</tbody>
</table>

Plus 10 additional comparisons not pertinent to the investigation.

### Table 7.8  Tukey post-test result for SR concentration response, surface CB$_1$

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>-6 vs. Vehicle</td>
<td>51.5044</td>
<td>7</td>
<td>9.4740</td>
<td>0.0002</td>
<td>Yes</td>
</tr>
<tr>
<td>-7 vs. Vehicle</td>
<td>50.2710</td>
<td>7</td>
<td>9.2472</td>
<td>0.0002</td>
<td>Yes</td>
</tr>
<tr>
<td>-8 vs. Vehicle</td>
<td>44.7739</td>
<td>7</td>
<td>8.2360</td>
<td>0.0004</td>
<td>Yes</td>
</tr>
<tr>
<td>-9 vs. Vehicle</td>
<td>19.5866</td>
<td>7</td>
<td>3.6029</td>
<td>0.2003</td>
<td>No</td>
</tr>
<tr>
<td>-10 vs. Vehicle</td>
<td>7.1939</td>
<td>7</td>
<td>1.3233</td>
<td>0.9614</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>-12 vs. Vehicle</td>
<td>7.1663</td>
<td>7</td>
<td>1.3182</td>
<td>0.9621</td>
<td>Do Not Test</td>
</tr>
</tbody>
</table>

Plus 15 additional comparisons not pertinent to the investigation.

### Table 7.9  Tukey post-test result for SR upregulation in HEK cells

<table>
<thead>
<tr>
<th>Factor</th>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface</td>
<td>Vehicle vs SR</td>
<td>36.4571</td>
<td>2</td>
<td>10.2376</td>
<td>0.0005</td>
<td>Yes</td>
</tr>
<tr>
<td>Intracellular pool</td>
<td>Vehicle vs SR</td>
<td>2.3504</td>
<td>2</td>
<td>0.66</td>
<td>0.656</td>
<td>No</td>
</tr>
<tr>
<td>Total</td>
<td>Vehicle vs SR</td>
<td>34.9355</td>
<td>2</td>
<td>9.8103</td>
<td>0.0005</td>
<td>Yes</td>
</tr>
<tr>
<td>Surface + Intracellular pool</td>
<td>Vehicle vs SR</td>
<td>43.552</td>
<td>2</td>
<td>12.2299</td>
<td>0.0003</td>
<td>Yes</td>
</tr>
</tbody>
</table>

### Table 7.10  Tukey post-test result for SR upregulation in Neuro2a cells

<table>
<thead>
<tr>
<th>Factor</th>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface</td>
<td>Vehicle vs SR</td>
<td>23.3972</td>
<td>2</td>
<td>4.3878</td>
<td>0.016</td>
<td>Yes</td>
</tr>
<tr>
<td>Intracellular pool</td>
<td>Vehicle vs SR</td>
<td>3.8172</td>
<td>2</td>
<td>0.7158</td>
<td>0.6275</td>
<td>No</td>
</tr>
<tr>
<td>Total</td>
<td>Vehicle vs SR</td>
<td>28.4024</td>
<td>2</td>
<td>5.3264</td>
<td>0.0064</td>
<td>Yes</td>
</tr>
<tr>
<td>Surface + Intracellular pool</td>
<td>Vehicle vs SR</td>
<td>27.2144</td>
<td>2</td>
<td>5.1036</td>
<td>0.0079</td>
<td>Yes</td>
</tr>
</tbody>
</table>
### Table 7.11 Tukey post-test result for transient transfection methods, transfection efficiency

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>A vs B</td>
<td>44.7541</td>
<td>3</td>
<td>7.6437</td>
<td>0.0007</td>
<td>Yes</td>
</tr>
<tr>
<td>B vs C</td>
<td>8.3327</td>
<td>3</td>
<td>2.0127</td>
<td>0.3633</td>
<td>No</td>
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<tr>
<td>A vs C</td>
<td>36.4214</td>
<td>3</td>
<td>6.2205</td>
<td>0.0029</td>
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</table>

### Table 7.12 Dunn’s post-test result for transient transfection methods, cell counts

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Ranks</th>
<th>Q</th>
<th>P&lt;0.05</th>
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</thead>
<tbody>
<tr>
<td>No DNA vs A</td>
<td>6.5</td>
<td>1.5765</td>
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<tr>
<td>No DNA vs B</td>
<td>10.3333</td>
<td>3.3624</td>
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<tr>
<td>No DNA vs C</td>
<td>5.25</td>
<td>1.5595</td>
<td>Do Not Test</td>
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### Table 7.13 Tukey post-test result for Rab5 influence on basal CB₁

<table>
<thead>
<tr>
<th>Factor</th>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.05</th>
</tr>
</thead>
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<tr>
<td>Surface</td>
<td>N vs. W</td>
<td>29.7057</td>
<td>3</td>
<td>10.4449</td>
<td>0.0011</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>N vs. P</td>
<td>8.9616</td>
<td>3</td>
<td>3.151</td>
<td>0.1482</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>P vs. W</td>
<td>20.7441</td>
<td>3</td>
<td>7.2939</td>
<td>0.0059</td>
<td>Yes</td>
</tr>
<tr>
<td>Intracellular Pool</td>
<td>P vs. W</td>
<td>11.9682</td>
<td>3</td>
<td>4.2082</td>
<td>0.0591</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>P vs. N</td>
<td>11.8152</td>
<td>3</td>
<td>4.1544</td>
<td>0.0618</td>
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<tr>
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<td>N vs. W</td>
<td>0.153</td>
<td>3</td>
<td>0.05379</td>
<td>0.9993</td>
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<tr>
<td>Total</td>
<td>P vs. W</td>
<td>24.8792</td>
<td>3</td>
<td>8.7478</td>
<td>0.0025</td>
<td>Yes</td>
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<td>P vs. N</td>
<td>8.5917</td>
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<td>3.021</td>
<td>0.1662</td>
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<td>N vs. W</td>
<td>16.2875</td>
<td>3</td>
<td>5.7269</td>
<td>0.0175</td>
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<td>MFR</td>
<td>N vs. P</td>
<td>39.6288</td>
<td>3</td>
<td>10.3407</td>
<td>0.0037</td>
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<tr>
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<td>N vs. W</td>
<td>32.7072</td>
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<td>8.5346</td>
<td>0.0075</td>
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<td>W vs. P</td>
<td>6.9216</td>
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<td>1.8061</td>
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<td>Comparison</td>
<td>Diff of Means</td>
<td>p</td>
<td>q</td>
<td>P</td>
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<tr>
<td>Surface</td>
<td>N vs. W</td>
<td>12.9528</td>
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<td>6.4599</td>
<td>0.0146</td>
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<td>N vs. P</td>
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<td>3.2043</td>
<td>0.154</td>
<td>No</td>
</tr>
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<td>3.2555</td>
<td>0.1477</td>
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<tr>
<td>Intracellular Pool</td>
<td>P vs. W</td>
<td>9.6175</td>
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<td>4.7965</td>
<td>0.0446</td>
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<tr>
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<td>P vs. N</td>
<td>5.8254</td>
<td>3</td>
<td>2.9053</td>
<td>0.1964</td>
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<tr>
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<td>N vs. W</td>
<td>3.7921</td>
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<td>1.8912</td>
<td>0.438</td>
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<td>Total</td>
<td>P vs. W</td>
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<td>4.4827</td>
<td>0.0563</td>
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<td>P vs. N</td>
<td>0.5527</td>
<td>3</td>
<td>0.2757</td>
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<td>N vs. W</td>
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<td>MFR</td>
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<td>P vs. N</td>
<td>13.7084</td>
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<td>N vs. W</td>
<td>15.3627</td>
<td>3</td>
<td>3.4068</td>
<td>0.1013</td>
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</table>

Table 7.14  Tukey post-test result for Rab7 influence on basal CB₁
Derivation of secondary equation for MFR calculation

\[
\frac{\text{Surface}^*}{\text{Intracellular}^*}
\]

\[
\frac{\text{Total}^* - \text{Intracellular}^*}{\text{Intracellular}^*}
\]

\[
\frac{\text{Total}^*}{\text{Intracellular}^*} - \frac{\text{Intracellular}^*}{\text{Intracellular}^*}
\]

\[
\frac{100}{\text{Intracellular}^*} - \frac{1}{1}
\]

* raw values or normalised to the same value

$^5$ normalised to total expression for the same treatment (ie. total = 100%)

All assuming that values for surface, total and intracellular expression arose from the same simultaneous experimental handling (including immunocytochemistry protocol) and quantification with the same analysis parameters.
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