Delineating the Specificity of Cannabinoid Effects by Investigating Cannabinoid Receptor-1 Trafficking and Signaling

by

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DEDICATION

To my mother, my sister, and my niece.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Definition
2AG	2-arachidonoylglycerol
6545	AM6545
AEA	arachidonoylethanolamine
ALK	anaplastic lymphoma kinase
ALP	alprenolol
AM	methanandamide
β1	β1-adrenergic receptor
β2	β2-adrenergic receptor
BFA	Brefeldin A
BLT2	Leukotriene B4 Receptor Type 2
CB1	Cannabinoid receptor-1
CB1-KO	Cannabinoid receptor-1 knock-out
CBD	cannabidiol

CHX	cycloheximide
COPI	coatomer protein 1
CRAC	cholesterol recognition amino acid consensus
CRIP-1a	cannabinoid receptor interacting protein 1a
CNS	central nervous system
D2R	dopamine D2 receptor
DOR	delta opioid receptor
DSE	depolarization-induced suppression of excitation
DSI	depolarization-induced suppression of inhibition
GASP	GPCR-associated sorting protein
GLP-1R	glucagon-like peptide-1 receptor
GPCR	G protein-coupled receptor
HEK293	human embryonic kidney-293
ISO	isoproterenol
JWH	JWH-018
KOR	kappa opioid receptor
LHR	luteinizing hormone receptor
LSD	lysergic acid diethylamide

MCD	methyl-b-cyclodextrin
MOR	μ-opioid receptor
PDZ	post-synaptic density-95/disc large tumor suppressor/zonula occludens-1
PKA	protein kinase A
PKC	protein kinase C
PKC	protein kinase D
SR	rimonabant
THC	delta-9-tetrahydrocannabinol
V2R	vasopressin 2 receptor
VEE	very early endosome
WIN	WIN 55,212-2
WIN-3	WIN 55,212-3

ABSTRACT

Pharmaceuticals target specific proteins within the body to produce desired therapeutic outcomes, and the G-protein-coupled receptor (GPCR) family represents the most common type of pharmaceutical target. Traditional models of GPCR function suggest that receptors are only active at the cell surface, however, multiple GPCRs have demonstrated activation at both surface and intracellular locations. This illuminates the possibility of GPCR spatial signaling, whereby a single GPCR can exhibit differential signaling outcomes based on its cellular localization. Therefore, GPCR trafficking could be targeted as a new pharmacological approach for modulating GPCR activity. In this thesis, I investigate cannabinoid receptor-1 (CB1) trafficking to determine how it may influence the specificity of cannabinoid-mediated effects.

CB1 is a G_i-coupled GPCR that is widely expressed throughout the central nervous system (CNS). At both excitatory and inhibitory presynaptic terminals, CB1's G_i-coupling reduces intracellular calcium concentrations in presynaptic neurons, and this regulates synaptic release of neurotransmitters. It is suggested that CB1's unique physiological function underlies its broad therapeutic potential for treating neurological disorders. However, the underlying trafficking mechanisms of CB1's axonal polarization remain unknown. It is well established that CB1 trafficking exhibits a high degree of constitutive recycling, yet CB1 does not recycle after agonist stimulation. Chapter 2 identifies biosynthetic trafficking as a key regulator of CB1 surface expression. Specifically, 2AG, an endogenous cannabinoid, upregulates biosynthetic trafficking of CB1 to the cell surface. This highlights a recycling-independent mechanism by which cells are resensitized to cannabinoids after acute agonist stimulation.

Cannabinoid agonists produce many similar effects in vitro and in vivo, but there are some effects are not entirely mediated by CB1. Chapter 3 characterizes novel biological effects of WIN 55,212-2 (WIN), a synthetic cannabinoid that has been used to investigate the endocannabinoid system, extensively. This chapter demonstrates that WIN disrupts the Golgi apparatus and microtubules in cultured cells, independent of CB1. This observation signifies a need for re-interpretation of previous WIN-based studies, particularly those that have speculated the functions of CB1 and the endocannabinoid system.

Overall, the work presented in this thesis highlights GPCR trafficking as crucial element of pharmacology research. Specifically, investigating CB1 trafficking helped establish a cellular mechanism by which CB1 activity is regulated within the CNS. Additionally, investigating how cannabinoids differentially regulate CB1 trafficking provided significant insight into the use of WIN as a pharmacological tool for studying the endocannabinoid system.

CHAPTER 1: Mechanisms of Selective GPCR Localization and Trafficking

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Abstract

G protein–coupled receptors (GPCRs), which share many structural and functional similarities, also share many mechanisms that traffic them between compartments. However, the Cannabinoid Receptor-1 (CB1) does not display traditional paradigms of GPCR trafficking. This raises the question of how the trafficking of individual GPCRs is selectively regulated. This is critical when investigating CB1's ability to regulate cellular function, as GPCR trafficking to different membrane compartments has recently emerged as a determinant of spatial signaling profiles. Here, we will discuss CB1 in the context of recent studies addressing the mechanisms that contribute to selectivity in endocytic and biosynthetic trafficking.

Introduction

The regulation of signaling by membrane trafficking has traditionally been attributed to trafficking's role in controlling the number of signaling receptors on the cell surface (Sorkin and von Zastrow, 2009). For G protein-coupled receptors (GPCRs), the largest single family of signaling receptors (Pierce et al., 2002), the removal of activated receptors from the cell surface by endocytosis and recovery of receptors on the surface by either recycling of internalized receptors or delivery of new receptors control the strength of response to extracellular ligands (Hanyaloglu and von Zastrow, 2008; Hausdorff et al., 1990). Recent studies, however, have highlighted more complex aspects of how trafficking regulates signaling. One is that GPCRs can signal from a variety of intracellular compartments (Sposini and Hanyaloglu, 2018; Lobingier and von Zastrow, 2019). Another is that mechanisms that regulate GPCR trafficking are heterogeneous, allowing selective control over the location and trafficking of individual GPCRs (Hanyaloglu and von Zastrow, 2008). These aspects have highlighted a new idea that the primary role of trafficking might be to move specific GPCRs between specific signaling complexes on different membrane domains, as opposed to simply regulating cell surface receptors (Crilly and Puthenveedu, 2020; Calebiro and Koszegi, 2019).

Cannabinoids and the Endocannabinoid System

Over the last three decades, the endocannabinoid system has emerged as an exciting research topic. This is largely attributed to the broad therapeutic implications of phytocannabinoids, naturally occurring compounds found in the marijuana plant. Anecdotal evidence suggests that delta-9-tetrahydrocannabinol, more commonly known as THC, has strong analgesic, anxiolytic, and anti-depressant properties, but its therapeutic potential is limited by the

accompanying psychoactive properties. There is, however, recognized therapeutic value of cannabidiol, or CBD, a phytocannabinoid that lacks psychoactive properties. Extensive preclinical and clinical research investigating CBD pioneered the development of Epidiolex, the first FDA-approved CBD therapeutic indicated for severe forms of childhood epilepsy (Devinsky et al., 2016; Devinsky et al., 2017; Devinsky et al., 2018; Jones et al., 2009; Jones et al., 2012; Kaplan et al., 2017; Szaflarski et al., 2018). To date, over 100 phytocannabinoids have been identified (Hill et al., 2012), and each could potentially have a unique profile of therapeutic possibilities, but we do not fully understand how these compounds produce their effects.

Marijuana is a classified as a Schedule I substance under the Controlled Substances Act, which means it has no established health benefit and a high potential for abuse (Mead, 2019). For context, heroin and LSD are also Schedule I substances. As previously stated, there is an abundance of anecdotal evidence that implicates cannabinoid compounds as powerful therapeutics, but federal regulations make it extremely difficult to investigate these claims in research settings. Since 2012, 19 states in the US have legalized marijuana for recreational purposes, and this number will continue to rise over the next decade. Accordingly, the breadth of therapeutic implications derived from anecdotal reports will continue to expand, but a comprehensive evaluation of these claims will not be possible as long as marijuana is a Schedule I substance.

Neurophysiology of The Cannabinoid Receptor-1

In 1988, the cannabinoid receptor-1 (CB1) was discovered as the pharmacological target THC (Devane et al., 1988). Since its discovery, mechanisms of CB1's physiology have been identified as regulatory components of cellular homeostasis. CB1 is broadly expressed within the central nervous system, but the particularly high expression in the amygdala, hippocampus, and

prefrontal cortex allows for regulation of neuronal processes such as anxiety, memory, and reward, respectively (dos Santos et al., 2020). It is suggested that the varied therapeutic implications of cannabinoids can be attributed to CB1's ability to modulate synaptic transmission. Specifically, CB1 exhibits presynaptic axonal polarization, which is defined as a preferential localization of receptors at axons over somatodendritic surfaces. This occurs at both GABAergic and glutamatergic synapses, and upon neuronal depolarization CB1 tapers the release of neurotransmitters (Figure 1.1). This process, defined as depolarization-induced suppression of inhibition and excitation (DSI and DSE, respectively), is unique to CB1, as it has not been observed with any other GPCR (Kreitzer & Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson & Nicoll, 2001). Although DSI and DSE are established components of neurological function, the underlying mechanisms by which CB1 modulates neurological states requires further investigation.

The Cannabinoid Receptor-1 as a Model GPCR

CB1 represents a physiologically relevant and pertinent model for investigating complex aspects of GPCR trafficking. At steady-state, CB1 is localized to both surface and intracellular compartments, while also displaying a high degree of constitutive trafficking (Leterrier et al., 2006; McDonald et al., 2006; Grimsey et al., 2010; Leterrier et al., 2004). Well-studied GPCRs such as the β2-adrenergic receptor and μ-opioid receptor have been fundamental to establishing traditional models of GPCR trafficking, but CB1 does not match these models and the physiological significance of this difference remains to be determined. In this review, we will discuss recent studies on endocytic and biosynthetic trafficking of GPCRs, focusing on example

mechanisms that provide specificity in the midst of shared mechanisms and the overall implications for CB1 activity.

Endocytic trafficking of CB1 and related GPCRs

The mechanisms of GPCR endocytosis and post-endocytic trafficking after receptor activation, which are common features of many GPCRs, have been exhaustively addressed in several reviews (Hanyaloglu and von Zastrow, 2008; Bowman and Puthenveedu, 2015; Bahouth and Nooh, 2017; Weinberg and Puthenveedu, 2019). We will discuss recent findings on receptor interactions and signaling pathways that provide selectivity within these mechanisms.

Selectivity in endocytosis of GPCRs

How the endocytosis of GPCRs is individually controlled has been a long-standing question, considering that the general mechanism is shared broadly across most GPCRs (Weinberg and Puthenveedu, 2019). Activated GPCRs undergo specific conformational changes that, in addition to catalyzing guanosine triphosphate (GTP) exchange on G proteins, allow GPCR kinases to phosphorylate the receptor C-termini. These phosphorylated C-termini are recognized by arrestins, which act as adapters that link receptors to the clathrin endocytic machinery (Tian et al., 2014; Gurevich and Gurevich, 2019A; Caron and Barak, 2019).

One aspect of this process that could be selective is receptor phosphorylation. Many GPCRs have multiple phosphorylation sites on its C-terminal tail, which are required for receptor internalization (Patwardhan et al., 2021; Gurevich and Gurevich, 2019B). For example, in the μ-opioid receptor (MOR), a phosphorylation cluster within residues 375-379 is the primary mediator of endocytosis (Lau et al., 2011; Arttamangkul et al., 2019), which might be driven mainly by

GRK2 in HEK293 cells (Bouley et al., 2020). C-terminal sites may be phosphorylated hierarchically by multiple kinases (Just et al., 2013; Duarte and Devi, 2020), suggesting that each GPCR could have a set of kinases that phosphorylate it and drives endocytosis. For example, the receptor tyrosine kinase anaplastic lymphoma kinase (ALK) associates with the dopamine D2 receptor (D2R), but not the closely related dopamine D1 receptor. An inhibitor of ALK blocks internalization of D2R, but not of D1R. ALK-mediated activation of protein kinase C g (PKCg) downstream of dopamine is required and sufficient for D2R internalization in HEK293 cells (He and Lasek, 2020). The exact ALK-dependent internalization mechanism is not clear, but PKCg may influence the phosphorylation patterns of D2R and target interactions between D2R and arrestin.

For the vasopressin 2 receptor (V2R), differences in phosphorylation at specific residues tuned the strength of arrestin interactions and regulate endocytosis. Mutation of Ser 357 or Thr 360 to alanines reduced arrestin binding as measured by co-immunoprecipitation but still retained enough binding to be visualized as membrane recruitment by microscopy. This reduced binding in the case of Ser 357 mutation was still sufficient for qualitatively similar levels of V2R and arrestin localization to endosomes. In contrast, reduced binding in the case of Thr 360 mutation abolished arrestin localization to endosomes, although its effect on V2R endocytosis was not directly measured (Dwivedi-Agnihotri et al., 2020). Similarly, a naturally occurring variant at Thr 282 for the angiotensin II receptor 1 induced a distinct conformation of arrestin upon binding, which was less stable but still supported endocytosis (Cao et al., 2020).

CB1 endocytosis is mediated by phosphorylation of its distal C-terminal sites. Within this region, there are six potential serine and threonine phosphorylation sites out of the 14 residues. Truncation of this distal region prevents CB1 endocytosis, which requires residues 460-463 (Hsieh

et al., 1999). However, preventing phosphorylation of only Thr 461 and Ser 463 does not sufficiently block endocytosis (Daigle et al., 2008). Instead, all six phosphorylation sites work in conjunction to regulate CB1 internalization via arrestin interactions (Daigle et al., 2008).

The second aspect of endocytosis that could be selective are "checkpoints" that exist after GPCR localization to endocytic domains (Figure 1.2). GPCR C-terminal tails contain specific sequences that interact with several components of the endocytic machinery. For example, a type I PDZ ligand on the C-terminus of the β2-adrenergic receptor indirectly links receptors to the actin cytoskeleton in clathrin-coated pits. This link delays the recruitment of dynamin, a GTPase that is required for membrane scission during endocytosis (Puthenveedu and von Zastrow, 2006). In contrast, PDZ-mediated interaction of mGluR1 and mGluR5, two metabotropic glutamate receptors, with the scaffold protein tamalin is essential for receptor endocytosis (Pandey et al., 2020). In this case, tamalin might link the receptors to motors via a scaffold protein S-SCAM, suggesting that it acts at a late step. An unrelated "bileucine" sequence on the C-terminal tail of MOR delays scission even after dynamin is recruited (Weinberg et al., 2017).

The same receptor might contain multiple discrete sequences that regulate endocytosis. The first intracellular loop of MOR contains specific lysines that are ubiquitinated by the ubiquitin ligase Smurf2. This ubiquitination, recognized by the endocytic accessory protein Epsin1, is required for endocytic scission (Henry et al., 2012). For the protease-activated receptor 1, ubiquitination-dependent recruitment of Epsin1 and the endocytic adapter AP-2 can induce receptor endocytosis in the absence of arrestins (Chen et al., 2011). The third intracellular loop of the β1-adrenergic receptor (β1) recruits endophilin, a BAR domain-containing protein that generates membrane curvature as part of the endocytic machinery, when linked to Giant

Unilamellar Vesicles. Endophilin, once recruited via interactions of the third loop with the endophilin SH3 domain, can generate membrane curvature on these vesicles (Mondal et al., 2021). Specific local protein interactions of individual GPCRs might therefore delay or facilitate their own endocytosis by modulating endocytic components. For CB1, the cannabinoid receptor interacting protein 1a (CRIP-1a) binds to CB1's distal C-terminal tail to decrease constitutive modulation of calcium signaling (Niehaus et al., 2007). Additionally, overexpression of CRIP-1a suppresses agonist-mediated endocytosis via dynamin and clathrin dependent mechanisms (Blume et al., 2016).

A third aspect is the selective interaction of GPCRs with membrane lipids. The third intracellular loop of the β1 receptor, described previously, electrostatically interacts with anionic phospholipids, which interfere with SH3 recruitment (Mondal et al., 2021). GPCRs might localize to micro-domains, such as lipid rafts or caveolae on the surface, often in a regulated manner (Patel et al., 2008; Briddon et al., 2018). Activation of the glucagon-like peptide-1 receptor (GLP-1R) in pancreatic beta cells redistributes the receptors to membrane nanodomains that contain the lipid raft marker flotillin (Buenaventura et al., 2019). When cholesterol was depleted by methyl-b-cyclodextrin, GLP-1R failed to redistribute to nano-domains and to internalize. Receptor palmitoylation and different agonists regulated this redistribution, raising the possibility that the process could be regulated by signaling.

The role that cholesterol interactions play could be specific for each GPCR. When cholesterol was depleted by statin drugs, 5-HT1A receptors (5-HT1AR) internalized, but the pathway switched from clathrin-mediated to caveolin-mediated endocytosis (Kumar and Chattopadhyay, 2020). Interestingly, when cholesterol was depleted to similar levels using methyl-b-cyclodextrin (MCD), 5-HT1AR still internalized via a clathrin-mediated pathway, although

postendocytic sorting was altered (Kumar and Chattopadhyay, 2021). CB1 localizes to lipid rafts on the plasma membrane. This distribution of receptors can be disrupted by both MCD-mediated cholesterol depletion and binding of the endocannabinoid, anandamide, to CB1 (Sarnataroa et al., 2005). Functionally, redistribution of CB1 outside of lipid rafts increases anandamide-mediated G protein signaling (Bari et al., 2005).

Several cholesterol-binding motifs, termed cholesterol consensus motifs, cholesterol recognition amino acid consensus (CRAC) motifs, or CARC motifs when they exist in reverse, have been identified in (Fantini et al., 2016; Fatakia et al., 2019). In many cases, the motifs have been functionally confirmed as being required for normal GPCR trafficking. A recent analysis of structural data across available GPCR structures, however, concluded that CRAC motifs are not predictive of cholesterol binding (Taghon et al., 2021). One potential way to reconcile these observations is that the motifs reflect potential hot spots of interactions (Sarkar and Chattopadhyay, 2020). Another way is to consider that lipid binding might be hierarchical, where allosteric changes caused by lipid binding on one site increases or decreases the affinity of other lipid-binding sites. In this context, it is important to note that the structural informatics (Taghon et al., 2021) was based largely on structures generated under conditions using synthesized lipids or detergents, which are different from in vivo environments where a full complement of lipids and proteins are present. Overall, much less is known about how lipids interact with GPCRs, compared with how proteins interact with GPCRs. This distinction is especially important for understanding the biology of CB1, as the endocannabinoids 2-arachidonoyl glycerol (2-AG) and anandamide are lipid-derived proteins (Lu and Mackie, 2016).

Selectivity in postendocytic trafficking of GPCRs

Internalized GPCRs typically have two fates once they are internalized and trafficked to the endosomal system. They may recycle back to the cell surface or may be degraded in the lysosome (Bowman and Puthenveedu, 2015; Bahouth and Nooh, 2017). Nutrient receptors such as the transferrin receptor are recycled largely by bulk membrane flow (Mayor et al., 1993), but GPCR recycling requires specific sequences on receptors. These sequences both restrict GPCRs from recycling by bulk flow and direct GPCRs to sequence-dependent recycling or degradation (Hanyaloglu and von Zastrow, 2008; Bahouth and Nooh, 2017). Mutating two protein kinase A (PKA) phosphorylation sites on β2 converts the receptor into a bulk recycling protein, suggesting that bulk sorting is hierarchically above sorting between sequence-dependent recycling and degradation (Vistein and Puthenveedu, 2013). Postendocytic sorting of the delta opioid receptor (DOR) to lysosomes occur via interactions with the GPCR-associated sorting protein, GASP (Whistler et al., 2002). Similarly, agonist binding targets postendocytic sorting of CB1 to lysosomes via GASP1 interactions, and disrupting this protein interaction upregulates CB1 recycling (Martini et al., 2006). At present, the factors that restrict GPCRs from accessing the bulk recycling pathway are not well-understood.

Spatial segregation of GPCRs in the endocytic pathway

The endolysosomal system is now recognized as a complex mix of partially overlapping membrane systems that constantly mature along the endocytic pathway (Figure 1.3). The current model is that endocytosed GPCRs pass through the very early endosome (VEE) to the early endosome (EE). The VEE is marked by APPL1 but devoid of Rab5 and EEA1, which mark the EE. The luteinizing hormone receptor (LHR) and the follicle-stimulating hormone receptor

(FSHR) are localized to the VEE after activation (Jean-Alphonse, et al., 2014). Many other GPCRs such as the prototypical β2 are localized mainly to EE after activation (Puthenveedu et al., 2010).

The steady-state segregation of GPCRs in distinct compartments likely represents receptor recycling from that compartment. LHR and FSHR are rapidly recycled from the VEE via interactions of receptor C-termini with the PDZ-containing protein GIPC. Disrupting PDZ-GIPC interactions decreases recycling and shifts the steady state distribution of LHR to the EE and later compartments (Jean-Alphonse, et al., 2014). Similarly, β2 is recycled from the EE by interactions of a PDZ ligand on its C-terminal tail with proteins in the actin-sorting nexin-retromer tubular domains of endosomes. Disrupting PDZ interactions decreases recycling and drives β2 into the late endosomal pathway to be degraded (Cao et al., 1999). For the atypical chemokine receptor 3, overexpression of RAMP3, a PDZ-containing member of a family of single-transmembrane proteins that associate with GPCRs, and NSF qualitatively changes receptor localization from Rab7 late endosomes to Rab4 early endosomes, after an hour of agonist treatment and 4 h of washout (Mackie et al., 2019). GPCRs in the EE may also be trafficked to a dedicated recycling endosome marked by Rab4 or Rab11, from which they can recycle. For CB1, receptors are endocytosed and trafficked to EE via Rab5 and the trafficked to Rab4-positive recycling endosomes for recycling (Leterrier et al., 2004). Receptor interactions with these specific components and localization depend on a slate of posttranslational modifications on the receptor, such as phosphorylation or ubiquitination (Patwardhan et al., 2021).

Regulation of GPCR sorting by signaling

Signaling pathways downstream of the same receptor (homologous) or other receptors (heterologous) could selectively regulate the rates of sorting and recycling of GPCRs by inducing

posttranslational modifications on select GPCRs. β2 activity reduces the rate of β2 recycling via receptor phosphorylation by PKA (Vistein and Puthenveedu, 2013). MOR activity, however, increases MOR recycling independent of PKA via phosphorylation at Ser 363 and Thr 370 by PKC downstream of receptor activation (Kunselman et al., 2019). PKC downstream of neurokinin-1 signaling also phosphorylates the same sites on MOR to increase MOR recycling and resensitization, allowing for cross-talk between these signaling pathways (Bowman et al., 2015). For the chemokine receptor CXCR4, however, PKC activation drives receptor degradation, suggesting that the same signaling pathway can affect receptors differently (Caballero et al., 2019). PKC phosphorylation of CXCR4 at Ser 324/ 325 recruits the ubiquitin ligase AIP4. PKC is sufficient, but not necessary, for CXCR4 degradation, suggesting that another kinase might phosphorylate one of these residues and recruit AIP4 (Caballero et al., 2019). Importantly, physiological systems might leverage postendocytic sorting mechanisms to fine tune the effects of receptor activation. Two endogenous ligands regulate the postendocytic fate of the kappa opioid receptor (KOR) differently (Kunselman et al., 2021). Dynorphin B causes KOR to rapidly recycle via Rab11, whereas Dynorphin A causes KOR to traffic to lysosomes for degradation. Interestingly, KOR can signal from lysosomes, which causes sustained signaling in comparison to when KOR recycles. Agonist-mediated internalization causes CB1 to traffic to the lysosome for degradation. However, CB1 also displays a high degree of constitutive recycling. Further research is required to determine how homologous and heterologous CB1 signaling may regulate receptor trafficking.

Biosynthetic trafficking of CB1 and related GPCRs

The folding and export of GPCRs from the endoplasmic reticulum is regulated by a variety of interacting proteins and by exogenous drugs that act as chaperones (Zhang and Wu, 2019; Doly and Marullo, 2015). In contrast, regulation of biosynthetic trafficking, which is defined as GPCR trafficking after ER export, is less understood. In this section, we will discuss recent data describing the heterogeneous mechanisms that regulate GPCRs transport from compartments after ER export (Figure 1.4).

Many "general" trafficking proteins, such as small monomeric GTPases and their interactors, have been implicated in GPCR export from the Golgi apparatus (Zhang and Wu, 2019). For example, the trafficking of α2B-adrenergic receptors depends on the Golgi-localizing, gamma-adaptin ear homology domain, ARF-binding (GGA) family of proteins and Rab26 (Zhang et al., 2016A; Zhang et al., 2016B; Wei et al., 2019). GGA1, 2, and 3 all interact with the third intracellular loop of α2B-adrenergic receptor, although by different mechanisms. Depleting any one of the GGAs causes a partial reduction in surface delivery of α2B-adrenergic receptor, suggesting that each of them is partially required. GGA3 binds an RRR motif in the loop, whereas GGA1 and 2 do not. GGA3 depletion reduces export also of α2C-adrenergic receptors, but not of α2A-adrenergic receptors. Rab26 also binds the same intracellular loop in a GTP-dependent manner, regulated by the putative GAP TBC1D6 (Wei et al., 2019). Unlike for GGA3, linear motifs on the receptor required for GGA1, GGA2, or Rab26 could not be identified by deletion studies, suggesting that they may bind a multipartite motif based on a specific conformation of the loop. Interestingly, an alternatively spliced variant of GGA1 lacks the hinge region of GGA1 that interacts with the α2B-adrenergic receptor, suggesting that isoform expression could provide selectivity (Zhang et al., 2019). As another example, the export of PAR2 from the Golgi requires

the activation of protein kinase D (PKD). In this case, PKD is activated by G$\beta\gamma$ trans-locationon to the Golgi after PAR activation, causing a feedback loop for repopulating the surface after receptor downregulation (Zhao et al., 2019). G$\beta\gamma$ and PKD are required for general TGN export (Diaz Anel and Malhorta, 2005), and whether other cargo molecules are also regulated downstream of PAR2 activation is not clear. Nevertheless, it is clear that some GPCRs use the predominant TGN export pathways to traffic to the cell surface.

Selective mechanisms that localize specific GPCRs without affecting trafficking in general have also been recently identified. The Leukotriene B4 Receptor Type 2 (BLT2) contains an unidentified sequence on its C- terminal tail, which enables it to interact with the scaffold protein LIN7C (Hara et al., 2021). A truncated BLT2 without this tail accumulates in the Golgi. But when LIN7C is depleted, BLT2 accumulates in intracellular compartments not restricted to the Golgi. In contrast, over-expression of the PDZ protein PIST localizes somatostatin receptor 5 and β1 receptor to the Golgi (Wente et al., 2005; Koliwer et al., 2015), presumably by interacting with the C-terminal PDZ ligand on the receptor.

The delta opioid receptor (DOR) provides a unique and interesting example of a GPCR whose Golgi localization is cell type specific and highly regulated. In neurons, newly synthesized DOR is retained in intracellular compartments that overlap with the Golgi, but in nonneuronal cells, DOR is efficiently expressed on the surface (Shiwarski et al., 2017A; Gendron et al., 2016). This Golgi localization is highly regulated by signaling. In the neuroendocrine PC12 cells, DOR is normally expressed at the cell surface, but a short exposure to Nerve Growth Factor, which inhibits phosphoinositide 3 kinase class 2 and reduces PI(3,4)P levels, induces Golgi localization of DOR (Shiwarski et al., 2017B). The current model for this retention is that in neurons or in NGF-treated PC12 cells, DOR is constantly retrieved to earlier compartments in the Golgi by

regulated interactions with the coatomer protein 1 (COPI) complex. DOR contains two atypical COPI-binding RXR motifs in its C-terminal tail (Shiwarski et al., 2019), which are required and sufficient for regulated Golgi localization. DOR contains additional canonical COPI-binding motifs in the second and third intracellular loops (St. Louis et al., 2017), which could contribute to a basal level of intracellular DOR. At present, whether these interactions are regulated is not known.

In contrast to DOR, endogenous cannabinoid receptor 1 (CB1) is localized to the late endosomal compartments and axonal surface in hippocampal neurons (Rozenfeld and Devi, 2008; Fletcher-Jones et al., 2019). The late endosomal localization could be because of the shunting of CB1R in the TGN to an adaptor protein 3-mediated export pathway (Rozenfeld and Devi, 2008). The deletion of helix 9 (H9) in the C-terminus caused CB1 to lose axonal polarization, but it was still delivered to the surface (Fletcher-Jones et al., 2019). This suggests that the receptor might be able to access multiple export pathways out of the TGN. The mechanism by which H9 regulates export is not known. The amphipathic nature of the helix might play a role, as amphipathicity of H8 was required for the export of apelin receptor from intracellular compartments and for efficient surface expression (Pandey et al., 2019).

Outside of specific adapters and interacting proteins, receptor oligomerization is an exciting possibility that could provide specificity to trafficking. For example, the transport protein RTP4 interacts with MOR and DOR and selectively increases expression of heteromers on the surface (Decaillot et al., 2008), without affecting individually expressed MOR and DOR or CB1 or dopamine 2 receptors (Fujita et al., 2019). Overall, the diversity of mechanisms that regulate Golgi retention and export suggest that GPCR delivery via the secretory pathway could be selectively regulated for individual GPCRs.

Conclusions

The subcellular location of GPCRs could be a master regulator of GPCR function, as the list of GPCRs capable of signaling from intracellular compartments is rapidly growing (Sposini and Hanyaloglu, 2018; Lobingier and von Zastrow, 2019; Crilly and Puthenveedu, 2020). Modulating signals from specific compartments, by either relocating receptors to the plasma membrane (Bowman et al., 2015; Shiwarski et al., 2017A) or specifically targeting signaling from endosomes (Jimenez-Vargas et al., 2020), has clear effects on signaling and behavior. As we develop sophisticated tools to study both the mechanisms of selective trafficking and localized signaling of GPCRs (Calebiro and Grimes, 2020; Halls and Canals, 2018; Maziarz et al., 2020), we will be able to generate a more precise understanding of spatial patterns of signaling for each member of this important family of signaling receptors.

The subcellular localization of CB1 presents an exciting opportunity to develop new models of GPCR trafficking and signaling. It is possible that CB1's varied localization throughout cells underlie spatial signaling paradigms that elicit unique cellular responses. Therefore, understanding the mechanisms that regulate CB1's trafficking could have profound implications for delineating the therapeutic potential of targeting the endocannabinoid system.

In this thesis, I will discuss CB1 trafficking and the unique effects of an extensively used synthetic cannabinoid agonist, WIN 55,212-2 (WIN). As I described in this chapter, CB1 trafficking is a complex mechanism of highly coordinated biological processes which regulate CB1's overall downstream signaling. In chapter 2, I will present two divergent paradigms of CB1 trafficking, whereby 2AG stimulation upregulates biosynthetic trafficking of CB1, while impairing constitutive recycling. In chapter 3, I will characterize WIN-mediated disruption of the Golgi

apparatus. This occurs independent of CB1, and it is the first time an effect of this magnitude has been reported for any cannabinoid. Finally, I will conclude this thesis by discussing the significance of this work as it relates to understanding the neurological implications of the endocannabinoid system.

Figures

Figure 1.1. CB1-Mediated Regulation of Neurotransmitter Release**.**

CB1 (green) exhibits axonal polarization in presynaptic neurons. Upon activation, downstream g_i-signaling of CB1 reduces intracellular calcium levels, thereby preventing neurotransmitter release (blue circles). The decreased availability of neurotransmitters at the synapse subsequently terminates activation of postsynaptic receptors (blue).

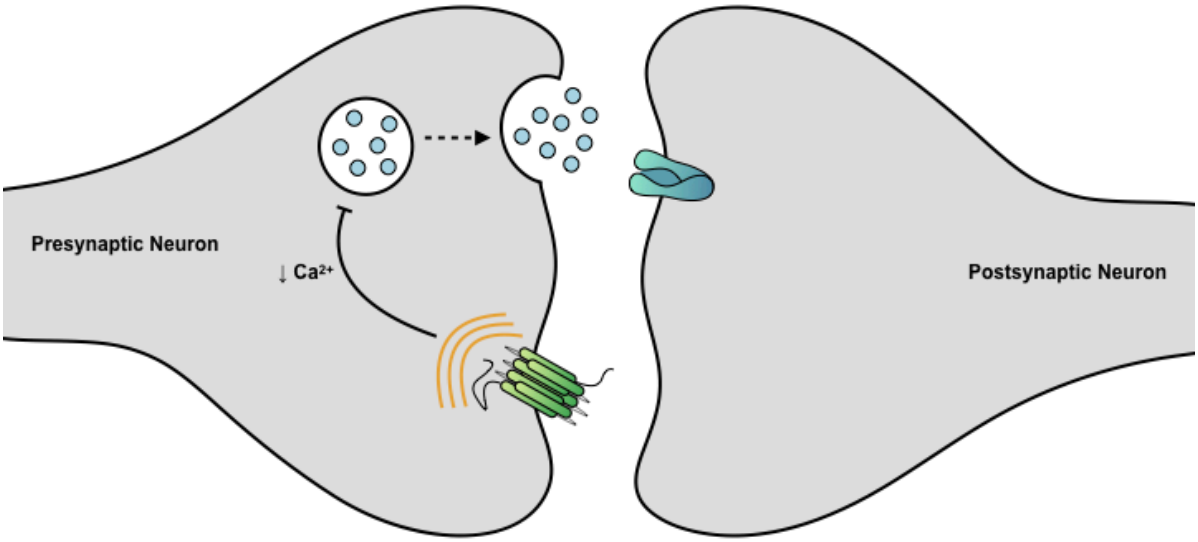


Figure 1.2. GPCR endocytosis is regulated by selective mechanisms.

GPCR endocytosis from the plasma membrane can be regulated at multiple steps. The 5- HT1AR can switch between clathrin-dependent or caveolin-dependent endocytosis depending on cholesterol levels in the plasma membrane, which suggests that GPCR endocytosis can be regulated by the local membrane environment. GPCR interactions with arrestin, a shared endocytic adapter, could be regulated by the slate of kinases that determine the phosphorylation patterns on the GPCR C-termini. The GPCR C-termini and cytoplasmic loops contain additional sequences that regulate later steps in endocytosis by interacting with structural scaffold proteins such as PDZ proteins or tamalin. Although these mechanisms are still not fully understood, newer methods including high resolution live cell microscopy and single molecule tracking may help us decipher the interplay between these factors, GPCRs, and the endocytic machinery.

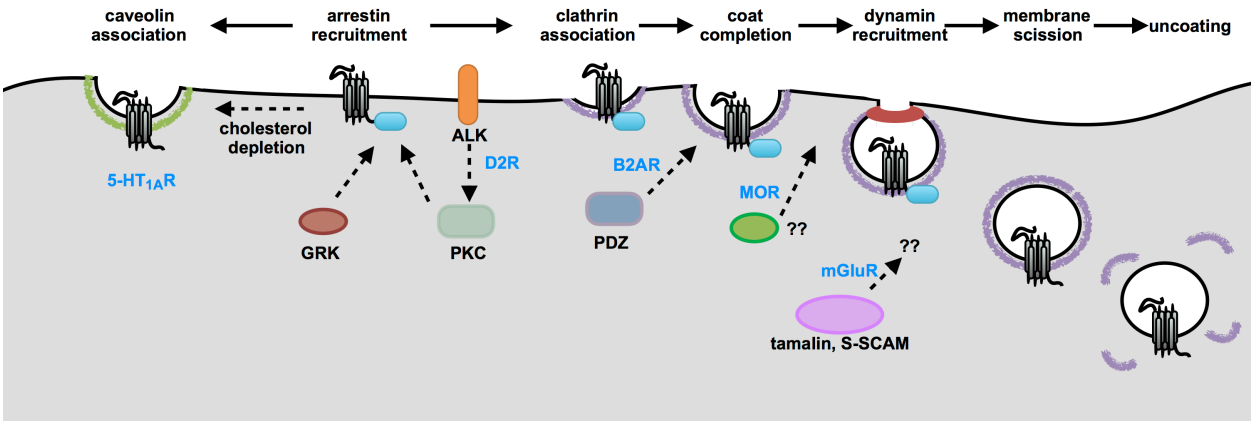


Figure 1.3. A sequential model for GPCR sorting throughout the endolysosomal network.

After internalization from the plasma membrane, GPCRs are sequentially transported through the VEE and EE, at which point they are sorted into the RE or the late endocytic/degradative pathway. These compartments are marked by specific biochemical components. GPCRs can interact with specific recycling trafficking proteins in these compartments that direct them to the recycling pathway. Selected examples of markers for compartments and GPCRs that recycle from them are shown. It is important to note that these compartments are depicted separately to denote where the majority of components are at steady state. In vivo, these compartments are likely to overlap significantly because of dynamic membrane exchange and maturation.

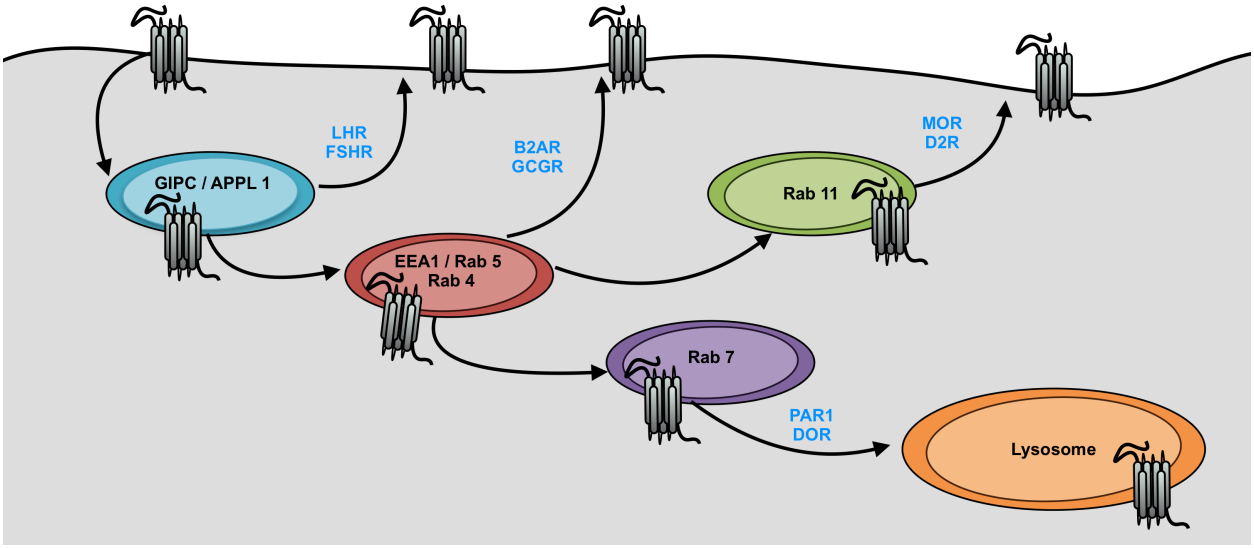
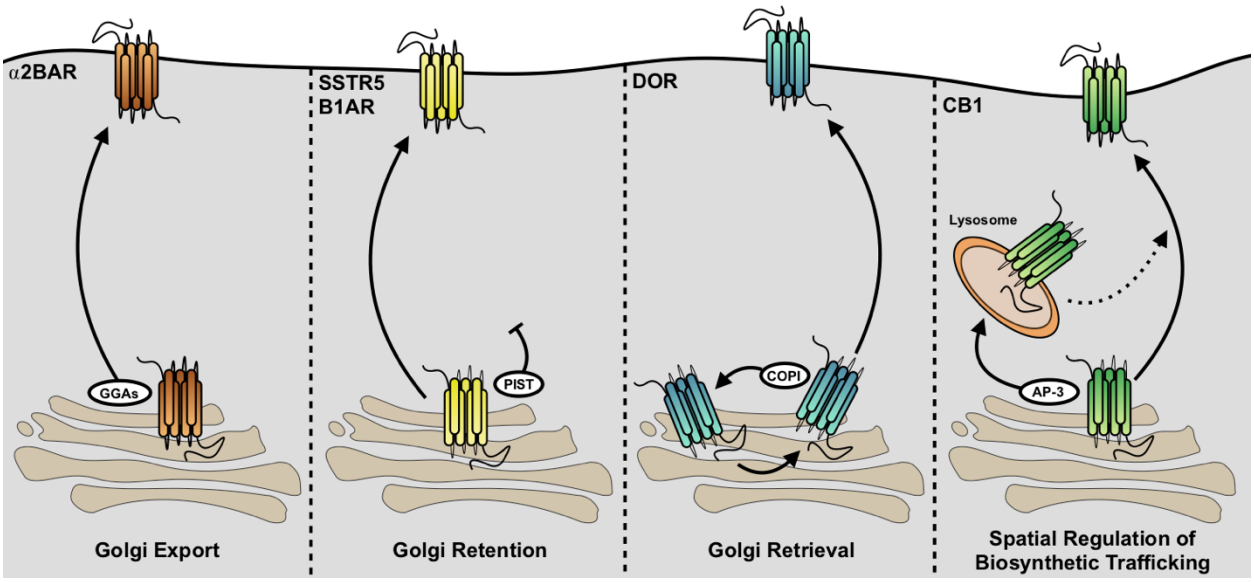


Figure 1.4. Post-Golgi trafficking of GPCRs can be regulated by diverse mechanisms.

Example pathways by which GPCR export can be regulated. GPCRs such as the α2B-adrenergic receptor and angiotensin II receptor type I are exported by interactions with GGA proteins. SSTR5 and β1 are retained in the Golgi via interactions with PIST, a PDZ-binding protein. DOR, on the other hand, is kept in the Golgi by constant retrieval via COPI interactions. CB1 is routinely trafficked to lysosomal compartments via AP-3 interactions, and disrupting these interactions redirects receptors to the plasma membrane. It is possible that additional pathways exist and that these pathways and interactions are relevant to different receptors in different cell types based on expression of components.



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CHAPTER 2: Feedback Regulation of Cannabinoid Receptor-1 Trafficking and Signaling

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Abstract

Cannabinoids primarily produce their effects by activating the cannabinoid receptor-1 (CB1), a Gi-coupled G protein-coupled receptor (GPCR) broadly expressed throughout the central nervous system (CNS). CB1 has a variety of neurological implications relating to mood, cognition, and neurodegenerative disorders. However, it is not clear how CB1's physiology influences neurological states. It is known that CB1 exhibits a high degree of constitutive trafficking and subcellular localization within neurons, which suggests that spatial organization may play a significant role in its overall function. Recent evidence supports GPCR trafficking throughout cells as an essential regulatory component of receptor function, therefore understanding the mechanisms of CB1 trafficking is crucial. Specifically, how cannabinoids influence the trafficking of CB1 requires further investigation. Here, we used a combination of sequential labeling with SNAP dyes and direct imaging of individual surface fusion events to investigate the mechanisms of CB1 trafficking in Human Embryonic Kidney-293 cells. We found that 2AG, an endogenous cannabinoid, halts constitutive recycling of CB1, while upregulating biosynthetic trafficking of CB1 to the cell surface. However, the synthetic cannabinoid AM356 does not upregulate biosynthetic trafficking. These findings uncover a novel mechanism of CB1 trafficking, whereby

endocannabinoids upregulate biosynthetic trafficking of CB1 to the cell-surface to repopulate receptors after agonist-mediated endocytosis.

Introduction

The Cannabinoid receptor-1 (CB1) is a member of the G protein-coupled receptor (GPCR) family, the largest class of therapeutic drug targets within pharmacology (Pierce et al., 2002). Its broad physiologically relevance is highlighted by the notion that it is one of the most abundantly expressed GPCRs in the CNS (Johnson & Lovinger, 2016). Like other Gi/o-coupled GPCRs, CB1 activation inhibits adenylyl cyclase activity (Howlett et al., 2004), however, its axonal polarization allows for dynamic regulation of neurotransmitter release (Castillo et al., 2012; Ohno-Shosaku & Kano, 2014). This modulation of neurotransmission is part of a highly coordinated retrograde signaling mechanism (Castillo et al., 2012; Ohno-Shosaku & Kano, 2014), and CB1's ability to suppress neuronal depolarization has been reported in numerous electrophysiological recordings (Kreitzer & Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson & Nicoll, 2001). For these reasons, CB1 can influence neuronal activity throughout the brain, and it has been implicated in the pathophysiology of Alzheimer's disease (Farkas et al., 2012) and Parkinson's disease (Lastres-Becker et al., 2001). The following study provides insight into CB1's broad neurological implications by investigating the cellular mechanisms that regulate its trafficking.

Canonically, GPCRs exhibit cell-surface localization and respond to extracellular stimuli such hormones, chemokines, protons, and neurotransmitters (Pierce et al., 2002), initiating a cascade of cellular responses though regulated G-protein signaling. However, emerging evidence suggest that these receptors not only signal from the cell surface, but from intracellular compartments as well (Crilly & Puthenveedu, 2020; Kunselman et al., 2021; Weinberg et al.,

2019), producing distinct signaling paradigms for a GPCR as determined by its localization upon activation. In addition to its unique presynaptic localization (Fletcher-Jones et al., 2019), CB1 exhibits robust intracellular localization at steady-state conditions (Leterrier et al., 2006; McDonald et al., 2006; Rozenfeld & Devi, 2008). Similar findings have been well-characterized in non-neuron cell types such as HEK293, CHO, and AtT-20 cells as well (Grimsey et al., 2010; Hsieh et al., 1999; Leterrier et al., 2004; Rinaldi-Carmona et al., 1998). The biological significance of this subcellular population of CB1 is not yet fully understood. Nevertheless, cannabinoids are typically membrane-permeable compounds (Pertwee, 2005) and endocannabinoids can access these subcellular populations (Brailoiu et al., 2011).

CB1 represents a pertinent model for investigating spatial paradigms of GPCR trafficking and signaling due to its steady-state cell-surface and intracellular localization, as well as the lipophilic nature of cannabinoid compounds. This "spatial paradigm" of GPCR activation has highlighted that neurotransmitter receptors, such as opioid receptors, can signal from intracellular Golgi and endosomal compartments (Shiwarski et al., 2017; Stoeber et al., 2018); similarly, the β1-adrenergic and β2-adrenergic receptors also signal from intracellular Golgi and endosomal compartments, respectively (Irannejad et al., 2013; Irannejad et al., 2017). It has been previously reported that endosomal CB1 recruits Gαi proteins in Neuro2A (Rozenfeld & Devi, 2008), and this suggests that subcellular signaling is possible for CB1 as well. However, a functional role for subcellular CB1 signaling remains to be determined.

Here, we used HEK293 cells to examine whether, and how, CB1 signaling regulates the surface expression of CB1 receptors. We found divergent constitutive and agonist-mediated paradigms of CB1 trafficking through a combination of experiments using SNAP-tagged and SpH-tagged receptors. CB1 constitutively recycles, however, internalized receptors do not recycle after

2AG-mediated endocytosis. Instead, new receptors repopulate the cell surface via 2AG-mediated upregulation of CB1's biosynthetic trafficking, but this is not observed with the synthetic cannabinoid AM. Our results introduce a novel model of CB1 trafficking whereby biosynthetic trafficking is upregulated in response to agonist stimulation, while constitutive trafficking is halted.

Materials and Methods:

Cell Culture and Reagents

All experiments performed in HEK293 cells (American Tissue Culture Collection, Manassas, VA) were cultured in DMEM high glucose (Thermo Fisher Scientific) + 10% fetal bovine serum (FBS, GIBCO). Transient expression of SNAP-B2 was obtained via construct transfection with Effectene (QIAGEN) according to the provided manufacture protocol. For stable cell lines (SNAP-CB1, SpH-CB1, SpH-MOR), transfection was followed by selection with Geneticin (Invitrogen). Cells were tested routinely for mycoplasma contamination. SNAP-Surface® Alexa® 488, SNAP-Surface® Alexa® 647, and SNAP-Cell® 647-SiR (300nM; New England Biolabs) were used to label SNAP-B2 and SNAP-CB1 in HEK293 cells.

Drugs used for treatment conditions were obtained and prepared as follows: 2AG purchased from Tocris Bioscience, and AM356 purchased from Cayman Chemical were prepared as 10mM stocks with ethanol. SR 141716A and AM6545 were purchased from Tocris Bioscience and prepared as 10mM stocks with DMSO. Isoproterenol purchased from Tocris Bioscience and Alprenolol purchased from Sigma-Aldrich were prepared as 10mM stocks with water.

Experimental Approach

Fixed-cell experiments were performed in 24-well glass bottom plates (Cellvis) coated with Poly-D-lysine (P6407; Sigma-Aldrich). After each experiment, cells were fixed with 4% paraformaldehyde (P6148; Sigma-Aldrich) for 15 minutes. Fixed cells were washed with 1x PBS for three minutes and then incubated in fresh 1x PBS for imaging.

Live-cell experiments were performed on 25mm coverslips (Thermo Fisher Scientific) coated with Poly-D-lysine (P6407; Sigma-Aldrich). For imaging, cells were incubated in Leibovitz (Thermo Fisher Scientific) + 1% fetal bovine serum (FBS, GIBCO).

Microscopy

Confocal and TIRF images were taken on a Nikon Eclipse Ti inverted microscope. A 60x/1.49 NA objective was used for confocal microscopy, and a a 100x/1.49 NA objective was used for TIRF microscopy. The images were acquired with an iXon+ 897 electron-multiplying charge-coupled device camera using Andor IQ software (Andor Technology).

Sequential-SNAP-Assay

SNAP-HEK293 cells were incubated with SNAP-labels for five minutes at 37℃, 5% CO2. For Sequential-SNAP labeling, HEK293 cells were incubated in the second SNAP-label immediately after the first SNAP-label was removed.

Statistical Analysis

All analysis was performed on GraphPad Prism 8, 2019. Prior to analysis, we determined the data that each group would be compared to the respective control condition. We used D'Agostino-Pearson test to analyze normal distribution of the data. For normally distributed data,

statistical significance for analysis comparing two experimental conditions was derived from unpaired t-tests, and for three or more conditions by ordinary one-way ANOVAs and multiple comparisons by a Dunnett's test. For data not normally distributed, statistical significance for analysis comparing two experimental conditions was derived from Mann Whitney tests, and for three or more conditions by Kruskal-Wallis test with multiple comparisons by a Dunn's test. All analyses are reported. For each graph representing SNAP experiments, n denotes the number of fields analyzed for each condition. For graphs representing SpH experiments, n denotes the number of cells analyzed for each condition for single-cell analysis. Statistical significance is denoted as follows: ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.001$.

Results

CB1 constitutively internalizes from the cell surface.

We first investigated how constitutive internalization of the Cannabinoid receptor-1 (CB1) compared to the β2 adrenergic receptor (β2), a G-protein coupled receptor (GPCR) that represents classical models of GPCR trafficking (Kunselman et al., 2021; Weinberg et al., 2019; Yudowski et al., 2006). To do this, HEK293 cells were transfected with either SNAP-CB1 or SNAP-β2. Stable SNAP-CB1 cell lines were generated, while SNAP-β2 experiments were conducted in transiently transfected cells. Using confocal microscopy and live-cell imaging, we examined dynamics of GPCR internalization by labeling receptors at the cell surface with a SNAP-surface alexa-488 dye. In unstimulated conditions, we noticed immediate internalization of SNAP-CB1, as indicated by the presence of internalized puncta, but we did not observe this with SNAP-β2 (Figure 2.1D). This suggest that CB1 constitutively internalizes in unstimulated conditions, which is in agreement with previous CB1 trafficking studies (Leterrier et al., 2004; Leterrier et al., 2006;

McDonald et al., 2006). Semi-automated quantification of the number of puncta shows that there are significantly more internalized puncta in SNAP-CB1 cells than there are in SNAP-β2 cells (Figure 2.1E).

GPCRs do not have to be in active confirmations at steady-state conditions, or have access to endogenous agonists, in order to be constitutively endocytosed (Scarselli & Donaldson, 2009; Mohammad et al., 2007; Wolfe et al., 2007). To test whether or not this was the case for CB1, we treated cells with either an inverse-agonist (SR) or a neutral antagonist (6545) for 15 minutes and quantified CB1 internalization across conditions. Fixed-cells were imaged using confocal microscopy, and we observed internalized SNAP-CB1 puncta in all conditions (Figure 2.1A). Our semi-automated quantification did not detect a significant difference in the number of internalized puncta when the SR or 6545 conditions were compared to control condition (Figure 2.1B). This suggests that constitutive internalization of CB1 cannot be attributed to the presence of endogenous ligands at steady-state. Additionally, this observation separates trafficking from previous studies which have determined that constitutive CB1 signaling can be inhibited by inverse agonist (Meye et al., 2012; Howlett et al., 2011; Sim-Selley et al., 2001).

Many, but not all, GPCRs internalize via clathrin-mediated endocytosis (Wolfe & Trejo, 2007). We investigated whether this was the case for constitutive internalization of CB1 by treating cells with the clathrin inhibitor, Pitstop. Over the course of 15 minutes CB1 steadily internalizes as puncta accumulates within the cell. However, when cells are pretreated with Pitstop the number of puncta within the cell does not increase, but instead remains constant throughout the duration of the 15-minute movie (Figure 2.1C). It is important to note that we still observe internalization in the Pitstop treatment condition. This suggests that constitutive internalization of CB1 is, at least in part, clathrin-independent.

CB1 constitutively recycles.

We next investigated whether CB1 exhibited constitutive surface delivery to maintain a stable surface expression as receptors are constitutively internalized. To do this, we transfected SpH-CB1 into HEK293 cells and generated a stable cell line. The SpH-tag fluoresces when exposed to neutral pH environments, such as extracellular media, but does not fluoresce when exposed to acidic pH environments, such as in endosomes (Martineau et al., 2017). This allows us to visualize receptors at the cell surface, as well as delivery of receptors to the cell surface, using TIRF microscopy (Flores-Otero et al., 2014; Weinberg et al., 2017; Kunselman et al., 2019). As exocytic vesicles fuse to the plasma membrane, SpH-tagged receptors packaged into those vesicles begin to fluoresce. The concentrated fluorescence appears as a bright spot that quickly diffuses across the cell membrane, and we define these exocytic events as a singular "puff" (Figure 2.2A). We compared constitutive surface delivery in our SpH-CB1 cell line to that of a stably expressed SpH-μ-opioid receptor (MOR) cell line. We used MOR as a control because SpH-MOR does not puff in the absence of agonist (Roman-Vendrell et al., 2012). Manual quantification of puff events determined that SpH-CB1 has significantly more puffs than SpH-MOR in unstimulated conditions (Figure 2.2B).

For CB1 to recycle, it must first be endocytosed. To test whether endocytosis was requires for the puffs, we treated SpH-CB1 cells with Pitstop, a recycling inhibitor, to determine if endocytosis was required for constitutive surface delivery. Additionally, we treated cells with Cycloheximide (CHX), a protein synthesis inhibitor, as a control for measuring biosynthetic trafficking. Manual quantification of puffs revealed that CHX decreased the number of puffs compared to control, although not significantly, while Pitstop significantly decreased the number of puffs compared to control (Figure 2.2C). Our results suggest that CB1 is constitutively recycled

to the cell surface in unstimulated conditions, and that a portion of constitutive surface delivery may be attributed to biosynthetic trafficking.

Cannabinoid agonists increase CB1 internalization but decrease CB1 recycling.

As we have demonstrated thus far, CB1 exhibits a considerable degree of constitutive trafficking, and it is of great interest to understand how cannabinoid agonists effect this constitutive activity. It is well-documented that cannabinoids produce pleiotropic effects (Morales & Reggio, 2020), therefore we sought to investigate how an endocannabinoid, 2AG, and synthetic cannabinoid, AM356 (AM), modulate CB1 trafficking. We treated SNAP-CB1 cells with either 2AG or AM for 15 minutes and quantified CB1 internalization across conditions. Fixed-cells were imaged using confocal microscopy, and we observed more internalized puncta in agonist-treated conditions (Figure 2.3A). This was confirmed by our semi-automated quantification whereby the number of puncta in agonist treatment conditions was significantly higher than what was detected in the control condition (Figure 2.3B). This is in agreement with traditional models of GPCR illustrating agonist-mediated internalization (Hamdan et al., 2007).

SNAP-labels bind covalently to SNAP-tags (Cole, 2013), and this allows for dynamic investigation of protein trafficking using live-cell imaging. Using SNAP-technologies, we were able to develop an assay for measuring GPCR-recycling of individual receptors in real time. We examined whether SNAP-CB1 immediately recycles back to the cell surface upon agonist-mediated endocytosis by treating cells with 2AG for 15 minutes, then incubating cells in a SR-washout for 15 minutes. The β2 receptor is known to recycle upon agonist-mediated internalization (Bowman et al., 2016; Puthenveedu et al., 2010; Yudowski et al., 2009), so we used SNAP-β2 as a control by treating cells with isoproterenol (ISO) for 15 minutes then incubating cells in an

alprenolol (ALP)-washout for 15 minutes. As expected, ISO induced SNAP-β2 internalization as determined by an increase in the number of puncta detected by semi-automated quantification, and the number of puncta decreased during the ALP-washout (Figure 2.3C). For SNAP-CB1, 2AG induced internalization, as determined by our semi-automated quantification, but the number of puncta continued to increase during the SR-washout (Figure 2.3C). This suggests that CB1 does not immediately recycle back to the cell surface upon agonist mediated endocytosis.

Because we did not see immediate recycling with SNAP-CB1, it was important to investigate how agonist stimulation affected SpH-CB1 recycling. We treated SpH-CB1 cells with 2AG for 10 minutes and measured the number of puffs before and after agonist treatment. Interestingly, manual quantification revealed a significant decrease in the number of puffs after 2AG treatment (Figure 2.3D), suggesting that agonist treatment blocks constitutive recycling of CB1.

CB1 is constitutively delivered to the cell-surface in a recycling-independent manner.

As previously mentioned, SNAP-technologies provide opportunities for developing innovative GPCR trafficking assays. The covalent nature of SNAP-labels and SNAP-tags allows for sequential labeling techniques to be implemented into fixed-cell assay design. For example, when HEK293 cells express a SNAP-tagged GPCR it is possible to incorporate multiple SNAP-labels into a singular assay. This allows for measurement recycling-independent surface delivery of GPCRs without the need for primary and secondary antibody labeling. Instead, two SNAP-surface labels could be used in the same assay without requiring permeabilization, creating a more flexible, yet precise, assay design.

An initial SNAP-surface label can be used to saturate receptors located at the cell-surface, thereby preventing any labeling of a secondary SNAP-surface label (Figure 2.4A), yet the same secondary SNAP-label can efficiently label SNAP-tags under normal circumstances (Figure 2.4B). We validated the principles of assay design through fixed-cell confocal microscopy labeling of SNAP-CB1 cells (Figure 2.4A and Figure 2.4B, bottom). We found that concentrations of SNAP-surface label greater than or equal to 100nM can efficiently saturate receptors at the cell surface and prevent labeling of a secondary SNAP-surface label at 300nM concentrations (Figure 2.4C). Alternatively, the secondary label could be SNAP-permeable, allowing for an assay design that measures both cell-surface and intracellular GPCR trafficking, which is particularly relevant for CB1 (Figure 2.4D).

Using what we have termed as the "Sequential SNAP" assay, we investigated mechanisms of recycling-independent CB1 surface delivery. For the previous examples of Sequential SNAP labeling, cells were fixed immediately after secondary labeling. However, it is possible to detect receptors that accumulate at the cell surface by extending the incubation time of secondary labeling (Figure 2.4E). We treated SNAP-CB1 cells with either Pitstop or CHX then incorporated 15 minutes of secondary labeling, SNAP-surface alexa-647, into the Sequential SNAP-surface assay. Semi-automated quantification of alexa-647 fluorescence detected a significant decrease in the CHX condition compared to control, but no difference between the Pitstop and control conditions (Figure 2.4F). This suggests that CB1 is constitutively delivered to the cell surface in a biosynthetic-dependent manner.

Cannabinoid agonists increase CB1 delivery to the cell-surface.

We next investigated how cannabinoid agonists affect recycling-independent trafficking of CB1 to the cell surface. To do this, we treated SNAP-CB1 cells with either 2AG or AM for 15 minutes and used the Sequential SNAP-surface assay to measure CB1 delivery to the cell surface, in the presence of agonist. It is important to note that CB1 undergoes rapid internalization (Leterrier et al., 2004), so the receptors detected can still be endocytosed over the course of the experiment. This became evident when SNAP-CB1 cells were imaged with confocal microscopy, showing internalized puncta, but minimal surface labeling, in both 2AG and AM conditions (Figure 2.5A). We used a semi-automated quantification approach to measure Alexa-647 fluorescence and detected a significant increase in the 2AG condition compared to control, but no significant difference between AM and control conditions (Figure 2.5B).

We sought to investigate the mechanism by which 2AG increases trafficking of CB1 to the cell surface. We modified our Sequential SNAP-surface assay to incorporate either a Pitstop or CHX pretreatment, which would block recycling and biosynthetic trafficking, respectively. The 2AG-mediated increase in alexa-647 fluorescence was no longer significant when compared to control for both the Pitstop and CHX conditions (Figure 2.5C). However, alexa-647 fluorescence does trend upward in the Pitstop condition compared to control, similar to cells treated with only 2AG. Together, these results suggest that 2AG, but not AM, increases trafficking of CB1 to the cell surface, and this increase requires biosynthetic trafficking of CB1.

Discussion

In this chapter, we show that agonist-mediated trafficking of CB1 does not follow the paradigms of constitutive trafficking. When constitutive endocytosis is blocked by Pitstop, there

is a subsequent decrease in CB1 recycling (Figures 2.2C). However, after agonist-mediated endocytosis, CB1 does not recycle when treated with an antagonist (Figure 2.3C). Instead, an agonist-mediated upregulation of biosynthetic trafficking repopulates CB1 to the cell surface, but this is only observed with the endocannabinoid 2AG (Figure 2.5B). In summary, 2AG upregulates biosynthetic trafficking of CB1, but inhibits recycling (Figure 2.6).

The spatial organization of CB1 within cells does not follow traditional models of GPCR localization or trafficking. CB1 is located intracellularly at basal conditions in hippocampal neurons (Leterrier et al., 2006; McDonald et al., 2006), Neuro2A cells (Rozenfeld & Devi, 2008), and HEK293 cells (Brailoiu et al., 2011; Leterrier et al., 2004), which suggests that a resident intracellular population of CB1 is required, at least in part, for its overall biological functions. It is known that CB1 constitutively internalizes from the cell surface (Gyombolai et al., 2013; Leterrier et al., 2004; Leterrier et al., 2006; McDonald et al., 2006), but it is unlikely that endocytic trafficking accounts for all intracellular populations. For example, lysosomal populations of CB1 originate from the Golgi apparatus and are trafficked directly to lysosomes upon biosynthesis (Rozenfeld & Devi, 2008); and mitochondrial CB1 exists within neurons (Bénard et al., 2012)., but it is not clear how CB1 is trafficked to this location. This supports the possibility of multiple trafficking paradigms for CB1. In this regard, CB1 is unique when compared to prototypical models of GPCR trafficking (Kunselman et al., 2021).

It is suggested that constitutive CB1 trafficking underlies mechanisms of axonal targeting, but this remains to be determined. Within neurons, GPCRs are typically trafficked to somatodendritic surfaces (Yudowski et al., 2006; Drake & Milner, 1999). However, CB1 is polarized to axonal membranes (McDonald et al., 2006; Fletcher-Jones et al., 2019; Leterrier et al., 2006), and is only transiently expressed at somatodendritic surfaces before undergoing rapid

internalization (Leterrier et al., 2006; McDonald et al., 2006). The postendocytic trafficking of CB1 is thought precede axonal targeting, though the current understanding of CB1 trafficking relies heavily on information gained from non-neuronal cultured systems. CB1 primarily internalizes via clathrin-mediated endocytosis in HEK293 cells (Flores-Otero et al., 2014; Leterrier et al., 2004), HeLa cells (Gyombolai et al., 2013), and AtT-20 cells (Hsieh et al., 1999), but in HEK293 cells it is possible for CB1 to internalize via caveolar-mediated endocytosis as well (Keren & Sarne, 2003). Again, supporting the possibility of multiple CB1 trafficking paradigms. This is particularly relevant to our results, as Pitstop, a clathrin inhibitor, does not fully prevent CB1 from internalizing (Figure 2.1). Additionally, Rab-GTPases regulate the post-endocytic trafficking of GPCRs (Esseltine & Ferguson, 2013), and constitutive CB1 trafficking requires Rab5 for internalization and Rab4 for recycling (Leterrier et al., 2004). Conversely, CB1 does not recycle under many circumstances (Hsieh et al., 1999; Rozenfeld, 2010; Grimsey et al., 2010), and this further prompts the need to further investigate multiple modes of CB1 trafficking.

Endocannabinoids are lipophilic compounds that readily cross membranes (Pertwee, 2006), enabling subcellular activation of CB1. Our results demonstrate a 2AG-mediated upregulation of biosynthetic trafficking (Figure 2.5), which could possibly be attributed to changes in the active state of subcellular CB1. It is known that GPCRs undergo c-terminal phosphorylation changes when they are activated, and this subsequently regulates their trafficking throughout cells (M. T. Drake et al., 2006; Bahouth & Nooh, 2017). For CB1, the distal regions of its c-terminus are required for internalization (Hsieh et al., 1999; (Daigle et al., 2008; Blume et al., 2016), and it cannot recycle in its phosphorylated state (Hsieh et al., 1999). Instead, CB1 at the surface undergoes endocytosis and postendocytic trafficking to lysosomes upon agonist stimulation (Martini et al., 2006; Martini et al., 2010). This suggests that an independent population of CB1

repopulates the cell surface, and we are not the first to suggest that this population is biosynthetic in origin (Grimsey et al., 2010). We believe that spatial signaling paradigms of CB1 regulate its biosynthetic trafficking in either a homologous or heterologous manner. Homologous regulation may occur if CB1's subcellular activation recruits accessory proteins that target it to the cell surface. Alternatively, heterologous regulation may occur if activation of CB1 at the cell surface diverts accessory proteins away from subcellular population, thereby enabling subcellular trafficking.

CB1 may produce different signaling outcomes depending on where activated receptors are signaling from, and this is an emerging topic in GPCR signaling (Weinberg et al., 2019; Crilly & Puthenveedu, 2020). This spatial regulation could either be attributed to differences in the active conformations of a receptor or differences in the availability and proximity of accessory proteins. However, we must further delineate the cellular outcomes of spatial signaling before we can fully interpret its physiological significance. It is well established that expression of CB1 at the surface influences its regulation of cellular function. However, it is not known whether subcellular CB1 works to repopulate receptor expression at the cell surface or if subcellular signaling facilities an entirely distinct cellular function. In conclusion, determining how CB1 trafficking is regulated throughout cells will advance our overall understanding of targeting CB1 and the endocannabinoid system for therapeutic purposes.

Figures

Figure 2.1. CB1 constitutively internalizes.

(A) HEK293 cells imaged with confocal microscopy showing fixed-SNAP-CB1 after 15-minute drug treatment conditions. Internalized puncta can be observed in control, SR [10μM], and AM6545 [10μM] conditions. Scale bar = 5μm. **(B)** Bar graph representation showing mean ± 95 CI of technical replicates. There is no statistically significant difference in the number of puncta counted between conditions. (ns, not significant; n = 3 biological replicates). **(C)** XY representation showing mean ± SEM of technical replicates of internalized puncta over time, while blue lines represent linear regression lines per condition. The slope for the control condition, Y = 0.6465*X + 15.63, is significantly non-zero ($p < 0.0001$; n = 3 biological), however, the slope for the Pitstop [30μM] condition, Y = -0.1235*X + 11.10, is not significantly non-zero ($p = 0.1423$; n = 3 biological replicates). Additionally, the slopes of between conditions are significantly different from each other ($p < 0.0001$). **(D)** HEK293 cells imaged with confocal microscopy showing live-SNAP-CB1 and live SNAP-B2. Internalized puncta can be observed in SNAP-CB1 cells, but not SNAP-B2 cells. Scale bar = 5μm. **(E)** Bar graph representation showing mean ± 95 CI of technical replicates. The number of puncta in SNAP-CB1 cells is significantly higher than the number of puncta in SNAP-B2 cells ($p < 0.0001$; $n \geq 10$).

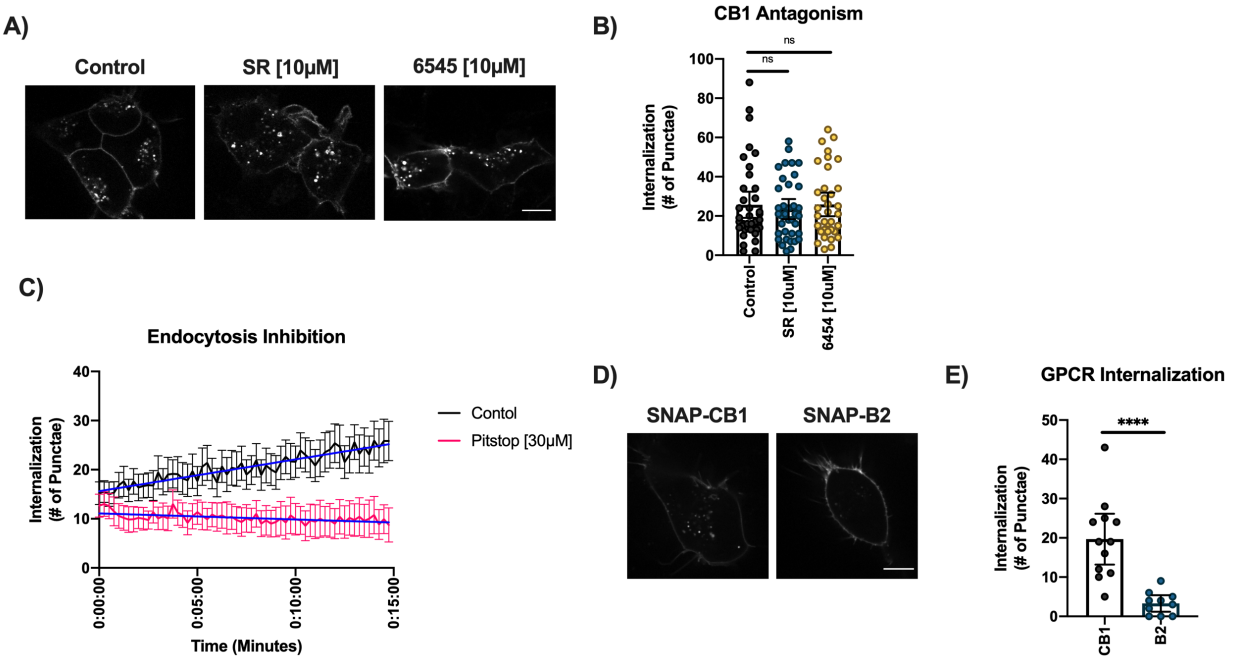


Figure 2.2. CB1 constitutively recycles.

(A) HEK293 cells imaged with TIRF microscopy showing live-SpH-CB1 delivery to the cell surface, and fluorescence surface plots matched to each image. Upon exocytic delivery of SpH-CB1 cargo, a concentrated fluorescence signal appears before quickly diffusing across the membrane. Each of these fusion events are defined as a single "puff". Scale bar = 2.5µm. **(B)** Bar graph representation showing mean ± 95 CI of constitutive GPCR surface delivery. SpH-CB1 exhibits significantly more puff activity than SpH-MOR (****, $p < 0.0001$; $n \geq 10$). **(C)** Bar graph representation showing mean ± 95 CI of SpH-CB1 surface delivery after inhibiting endocytosis and biosynthetic protein transport. A 30-minute pretreatment of Pitstop [30µM] significantly reduced puff activity compared to the control condition (****, $p < 0.0001$; $n \geq 8$), but a 2-hour pretreatment with CHX [10µM] did not (ns, not significant; $n \geq 9$).

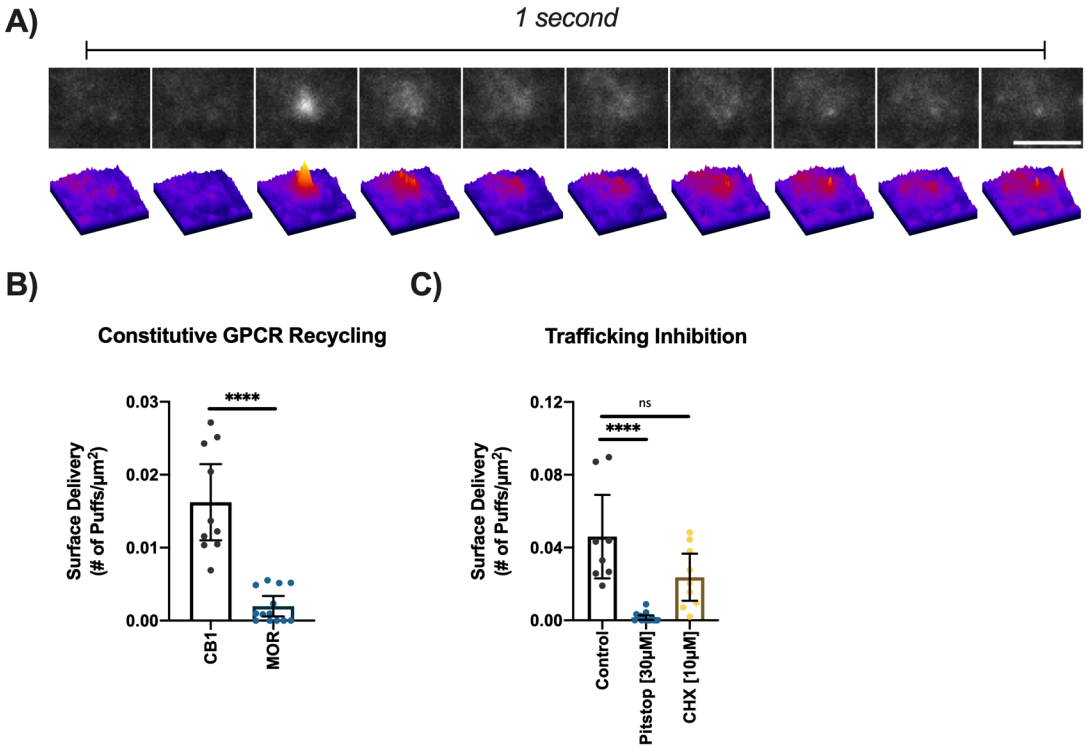


Figure 2.3. Cannabinoid agonists increase CB1 internalization but decrease CB1 recycling.

(A) HEK293 cells imaged with confocal microscopy showing fixed-SNAP-CB1 after 10-minute drug treatment conditions. Internalized puncta can be observed in control, 2AG [10μM], and AM [10μM] conditions, however, SNAP-CB1 is not present at the cell-surface in the control condition. Scale bar = 5μm. **(B)** Bar graph representation showing mean ± 95 CI of technical replicates. There are significantly more internalized puncta in the 2AG [10μM], and AM [10μM], treatment conditions compared to control (**, $p < 0.01$; ****, $p < 0.0001$; n = 3 biological replicates). **(C)** XY representation showing mean ± SEM of technical replicates of internalized puncta over time. For both SNAP-CB1 and SNAP-B2, the number of internalized puncta increases from baseline upon a 15-minute agonist treatment, 2AG [10μM] and ISO [10μM], respectively. The number of SNAP-CB1 puncta continues to rise after a media washout with the inverse-agonist SR [10μM], however, the number of SNAP-B2 puncta decreases after a media washout with the antagonist ALP [10μM]. (n = 3 biological replicates). **(D)** Bar graph representation showing mean ± 95 CI of SpH-CB1 surface delivery after agonist treatment. A 10-minute 2AG [10μM] treatment significantly reduced puff activity compared to the control condition (*, $p < 0.05$; n = 8).

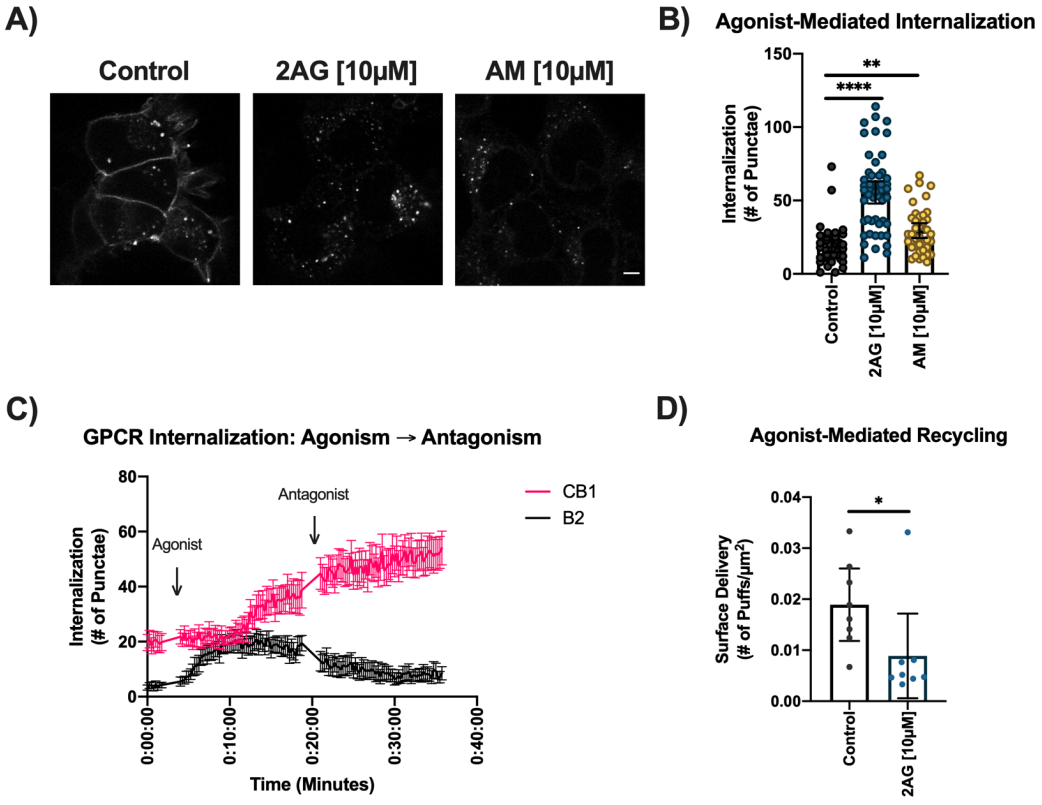
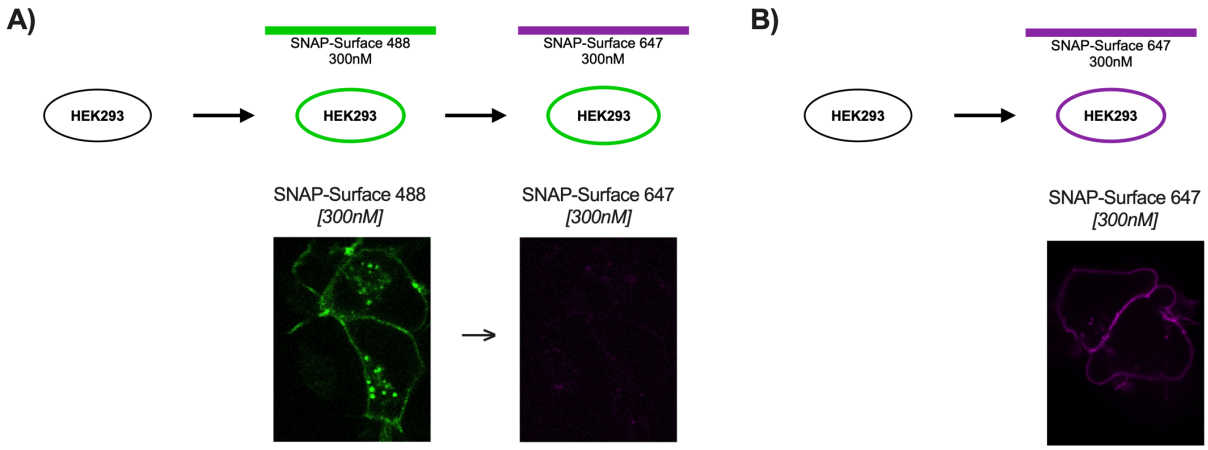
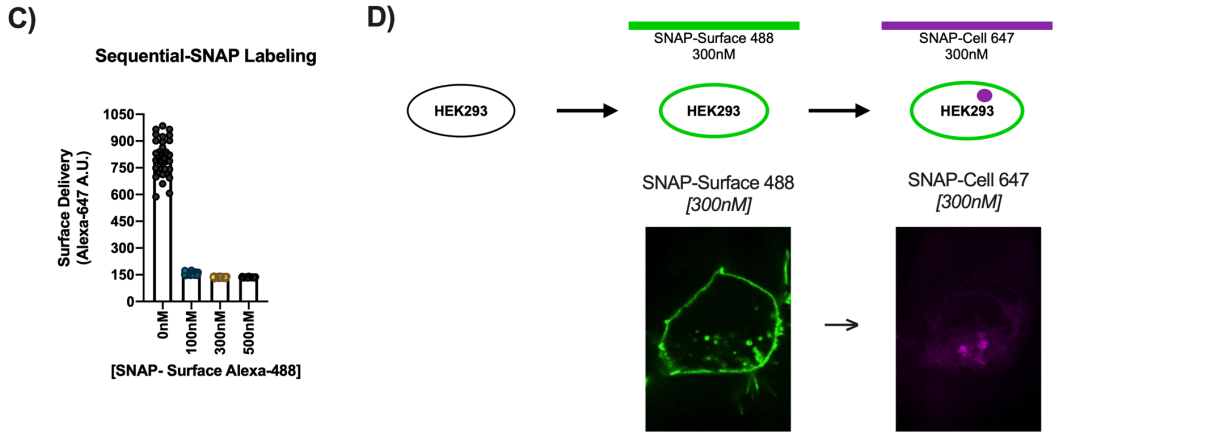


Figure 2.4. CB1 is constitutively delivered to the cell-surface in a recycling-independent manner.

(A) Schematic of the Sequential-SNAP labeling approach. SNAP-labels covalently bind to SNAP-tags. Therefore, one SNAP-surface label can be used to saturate SNAP-tags available at the cell-surface and prevent labeling of a second SNAP-surface label. HEK293 cells imaged with confocal microscopy showing fixed-SNAP-CB1 after Sequential-SNAP labeling. No alexa-647 fluorescence is detected when SNAP-Surface 647 labeling immediately follows SNAP-Surface 488 labeling. **(B)** Schematic of SNAP labeling. HEK293 cells imaged with confocal microscopy showing SNAP-Surface 647 efficiently labels under normal conditions. **(C)** Bar graph representation showing mean ± 95 CI of technical replicates. Concentrations of SNAP-Surface® Alexa® 488 ≥ 100nM sufficiently prevents SNAP-Surface® Alexa® 647 labeling (n = 3 biological replicates). **(D)** Schematic of the Sequential-SNAP labeling approach using SNAP-Cell® 647-SiR. One SNAP-surface label can be used to saturate SNAP-tags available at the cell-surface and prevent surface labeling of a SNAP-permeable label. However, the SNAP-permeable label pass through membranes and bind to intracellular SNAP-tags. HEK293 cells imaged with confocal microscopy showing fixed-SNAP-CB1 after Sequential-SNAP labeling. Alexa-647 fluorescence is detected intracellularly, but not at the cell surface. **(E)** Schematic of the Sequential-SNAP assay with a variable SNAP-Surface 647 labeling period. Coincubation of SNAP-Surface 647 with drug treatment can be used to measure changes in new receptor delivery over time. **(F)** Bar graph representation showing mean ± 95 CI of alexa-647 fluorescence after inhibiting endocytosis and biosynthetic protein transport. Using the Sequential-SNAP labeling approach, no significant difference in new receptor delivery was detected after 15 minutes between the control and 30-minute pretreatment Pitstop [30μM] condition (ns, not significant; n = 13 technical replicates). However, less new receptors delivery was detected in the 2-hour CHX [10μM] condition when compared to control (**, $p < 0.01$; $n \geq 13$ technical replicates).


A)
SNAP-Surface 488
300nM
SNAP-Surface 647
300nM
HEK293
HEK293
HEK293
SNAP-Surface 488
[300nM]
SNAP-Surface 647
[300nM]
B)
SNAP-Surface 647
300nM
HEK293
HEK293
SNAP-Surface 647
[300nM]


C)
Sequential-SNAP Labeling
Surface Delivery
(Alexa-647 A.U.)
1050
900
750
600
450
300
150
0
0nM
100nM
300nM
500nM
[SNAP- Surface Alexa-488]
D)
SNAP-Surface 488
300nM
SNAP-Cell 647
300nM
HEK293
HEK293
HEK293
SNAP-Surface 488
[300nM]
SNAP-Cell 647
[300nM]

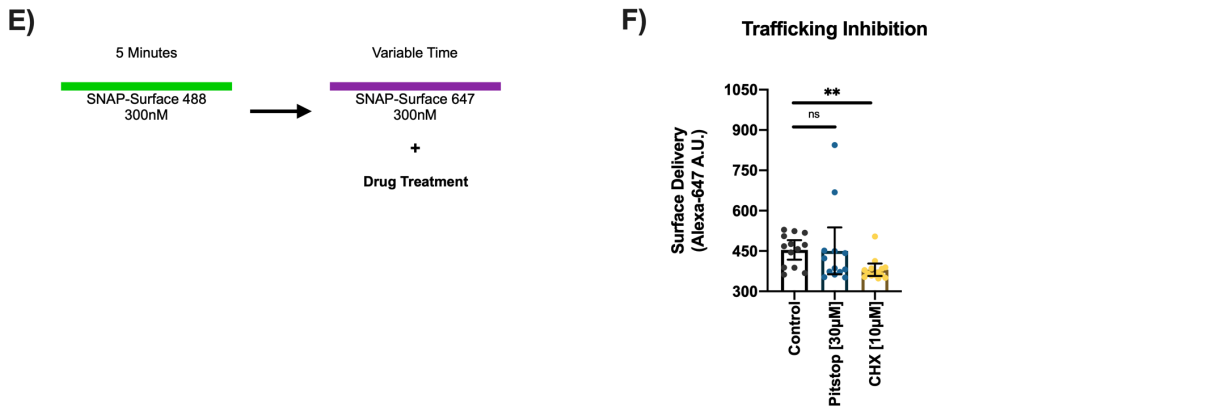

E)
5 Minutes
Variable Time
SNAP-Surface 488
300nM
SNAP-Surface 647
300nM
+
Drug Treatment
F)
Trafficking Inhibition
Surface Delivery
(Alexa-647 A.U.)
1050
900
750
600
450
300
**
ns
Control
Pitstop [30μM]
CHX [10μM]

Figure 2.5. Cannabinoid agonists increase CB1 delivery to the cell-surface.

(A) HEK293 cells imaged with confocal microscopy showing fixed-SNAP-CB1 after 30-minute drug treatment conditions. Alexa-647 fluorescence was present in all conditions. Scale bar = 5µm **(B)** Bar graph representation showing mean ± 95 CI of technical replicates. 2AG [10µM] treatment causes a significant increase in new receptor delivery compared to the control condition (****, $p < 0.0001$; n = 3 biological replicates), but AM [10µM] treatment does not (ns, not significant; n = 3 biological replicates). **(C)** Bar graph representation showing mean ± 95 CI of technical replicates. 2AG [10µM] treatment causes a significant increase in new receptor delivery compared to the control condition (**, $p < 0.01$; n = 3 biological replicates). However, 2AG [10µM] treatment does not significantly increase new receptor delivery compared to the control condition when HEK293 cells are pretreated with either Pitstop [30µM] for 30 minutes or CHX [10µM] for 2 hours (ns, not significant; n = 3 biological replicates).

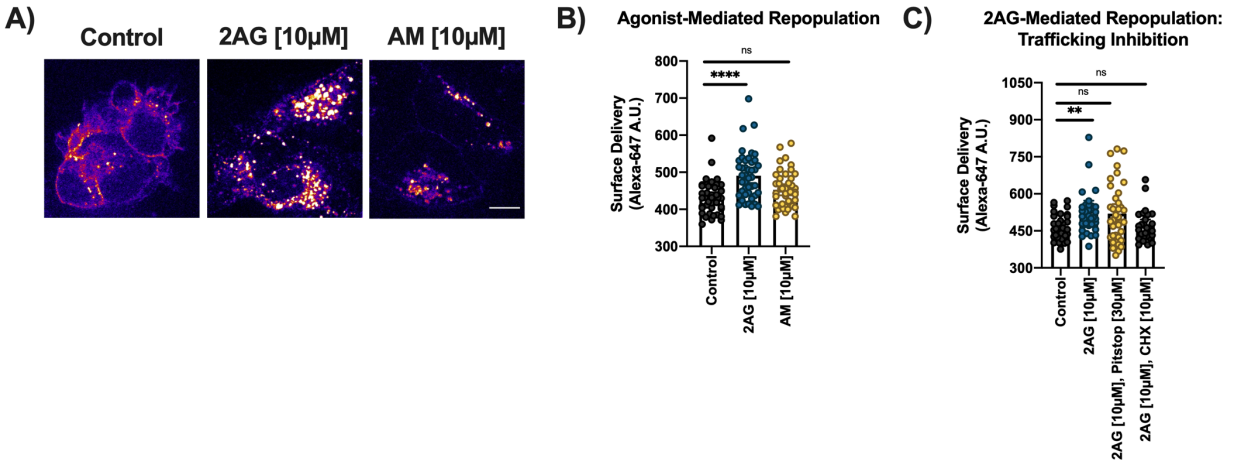
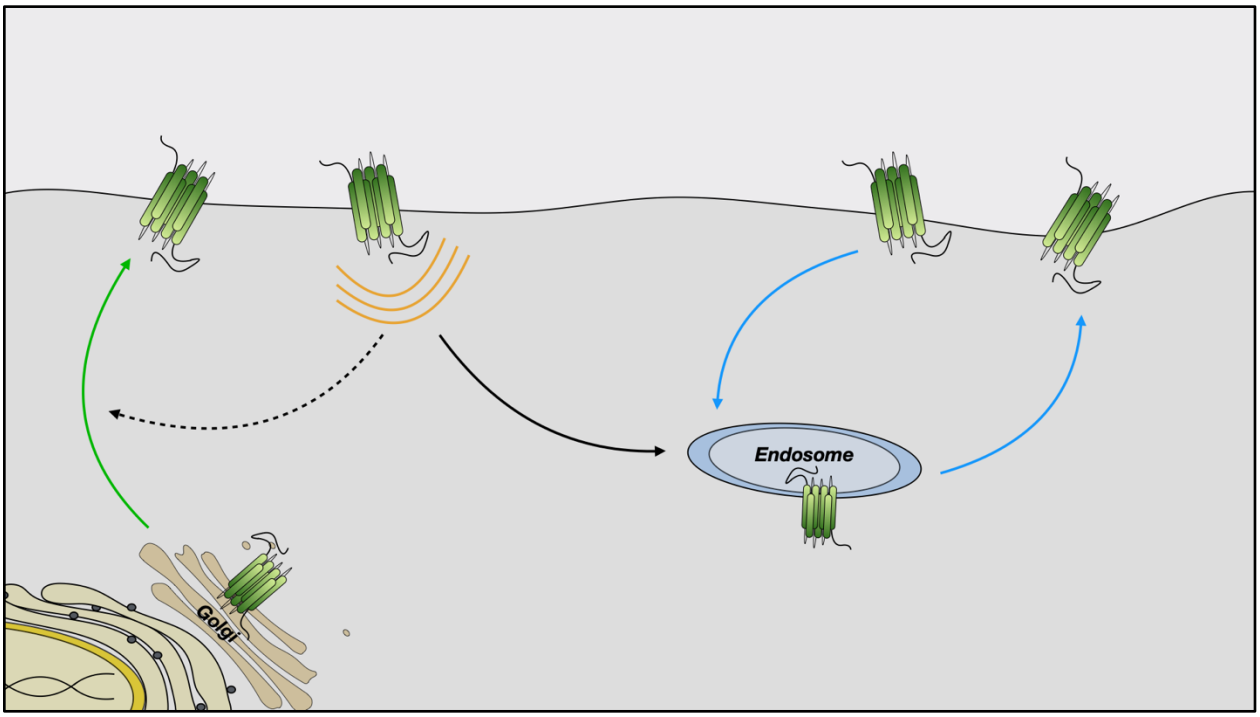


Figure 2.6. CB1 Trafficking Model.

CB1 is delivered to the cell surface via recycling and biosynthetic trafficking. Constitutive trafficking of CB1 (blue arrows) occurs as CB1 is endocytosed and recycled back to the cell surface. However, CB1 does not recycle following receptor activation (orange lines). Agonist stimulation upregulates CB1 endocytosis (black arrow), but not recycling. Instead, 2AG upregulates biosynthetic trafficking of CB1 from the Golgi to the cell surface (green arrow) in a recycling-independent manner.



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CHAPTER 3: The Synthetic Cannabinoid WIN 55,212-2 Can Disrupt the Golgi Apparatus Independent of Cannabinoid Receptor-1

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Abstract

The synthetic cannabinoid WIN 55,212-2 (WIN) is widely used as a pharmacological tool to study the biological activity of cannabinoid receptors. In contrast to many other cannabinoid agonists, however, WIN also causes broad effects outside of neurons, such as reducing inflammatory responses, causing cell cycle arrest, and reducing general protein expression. How exactly WIN causes these broad effects is not known. Here we show that WIN partially disrupts the Golgi apparatus at nanomolar concentrations, and fully disperses the Golgi apparatus in neuronal and non-neuronal cells at micromolar concentrations. WIN 55,212-3, the enantiomer of WIN, JWH-018, a related alkylindole, or 2-arachidonoylglycerol, an endocannabinoid, did not cause Golgi disruption, suggesting the effect was specific to the chirality of WIN. WIN treatment also perturbed the microtubule network. Importantly, WIN disrupted the Golgi in primary cortical neurons derived from mice where CB1 was genetically knocked out, indicating that the effects were independent of CB1 signaling. The Golgi dispersion could not be explained by WIN's action on Peroxisome Proliferator-Activated Receptors. Our results show that WIN can disrupt the Golgi

apparatus independent of CB1 in cultured cells. These effects could contribute to the unique physiological effects that WIN exhibits in neuronal behavior, as well as its role as an antiproliferative and anti-inflammatory agent.

Introduction

Cannabinoid ligands are versatile pharmacological agents. Cannabinoids primarily activate the Cannabinoid receptor-1 (CB1), a G protein-coupled receptor (GPCR) that plays a vital role in modulating neurotransmission in the central nervous system (CNS) and in the peripheral nerves. In the CNS, CB1's activation is part of a highly coordinated retrograde signaling mechanism (Castillo et al., 2012; Ohno-Shosaku and Kano, 2014), which suppressed neuronal depolarization as highlighted by numerous electrophysiological recordings (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Cannabinoid agonists are valuable therapeutics for addressing drug addiction, neuropathic pain, epilepsy and various psychiatric disorders (Jetly et al., 2015; Mücke et al., 2018; O'Connell et al., 2017; Segura et al., 2019; Smith et al., 2015).

The synthetic cannabinoid WIN55,212-2 (WIN) is a high affinity CB1 agonist that is widely used to investigate cannabinoid physiology. WIN's increased potency compared to the endogenous cannabinoids 2-arachidonoylglycerol (2AG) and anandamide (AEA), which are partial agonists for CB1 (Pertwee, 2005), oftentimes makes it a preferred compound used to study cannabinoid pharmacology in cells and animals (Chen et al., 2021; Martini et al., 2010; Sim-Selley and Martin, 2002). However, WIN is a particularly interesting cannabinoid agonist in this regard, as it shows unique pharmacological and physiological outcomes. These outcomes could be driven by WIN's unique chemical properties, including stereoisomer specificity (Emery et al., 2013). One notable physiological difference between WIN and other cannabinoids is observed in cancer

pharmacology, where WIN has been shown to cause robust anti-proliferative effects in oncogenic cells (Emery et al., 2013; Müller et al., 2017; Pellerito et al., 2014; Scuderi et al., 2011; Wasik et al., 2011). In addition, WIN has also been proposed to have anti-inflammatory and antiproliferative effects in a variety of settings (Marchalant et al., 2007; Marchalant et al., 2008; Wang et al., 2018; Zhao et al., 2010). These broad effects have generated interest in the role of synthetic cannabinoids like WIN in physiological systems outside of the CNS.

One overarching theme that is unique to WIN's actions at the cellular level is a reduction in the surface expression and secretion of proteins. For neurotransmitter receptors and their accessory proteins, this reduction can have profound effects on neurotransmission (Blume et al., 2013; Perdikaris et al., 2018). WIN down-regulates the surface expression of CB1 and $GABA_A$ subunits (Deshpande et al., 2011). This reduction might be partly due to a decrease in total protein expression via mRNA downregulation (Perdikaris et al., 2018; Tan and Cao, 2018). Similarly, in non-neuronal cells, WIN reduces the generation of inflammatory mediators in non-neuronal cells (Lowin et al., 2016), suggesting that the effects of WIN on surface expression and secretion of proteins could be general. However, whether WIN generally regulates protein trafficking, and whether WIN's effects on surface expression of proteins are via CB1 activation, are not fully known.

In this study, we used high-resolution fluorescence microscopy to explore the effects of WIN on components of the trafficking machinery in neuronal and non-neuronal cells. We found that WIN, but not its enantiomer WIN55,212-3, the related alkylindole JWH-018, or the endocannabinoid 2AG, partially disrupts the Golgi apparatus at nanomolar concentrations and completely disrupts the Golgi apparatus in both neuronal and non-neuronal cells at micromolar concentrations. WIN treatment disrupted the Golgi in neurons obtained from CB1 knock-out (KO)

mice, suggesting that this effect is CB1-independent. Our findings provide a potential mechanism by which WIN can reduce levels of select surface proteins and produce a broad range of physiological effects independent of CB1.

Materials and Methods

Cell Culture and Reagents

Experiments performed in HEK293 cells (American Tissue Culture Collection, Manassas, VA) were cultured in DMEM high glucose (Thermo Fisher Scientific) + 10% fetal bovine serum (FBS, GIBCO). E18 striatal neurons were obtained from BrainBits, LLC and cultured for 2 weeks following the recommended protocol. Cortical CB1-KO neurons were obtained from P0 mice and cultured for 2 weeks following the BrainBits, LLC recommended protocol. Cells in the lab are tested routinely for mycoplasma contamination. CB1 knockout mice were described in Ledent et al. (1999). SNAP-Cell® 647-SiR (300nM; New England Biolabs) was used to label SNAP-CB1 in HEK293 cells. Drugs used for treatment conditions were obtained and prepared as follows: 2AG was purchased from Tocris Bioscience and prepared as a 10mM stock with ethanol. WIN, GW6471 and GW9662 were purchased from Sigma-Aldrich (St. Louis, MO) and prepared as 10mM stocks with DMSO.

Experimental protocols

HEK293 cells were transfected with SNAP-CB1 and GPP130-GFP using Effectene (QIAGEN) according to the provided manufacture protocols. Stable cell lines were generated using selection with Geneticin (Invitrogen). Cell viability was assessed by labeling cells with Trypan Blue stain and manually counting the cells labeled using a counting chamber. Protein

secretion was estimated by washing cells with PBS, incubating cells in PBS for 1 hour, and estimating the amount of protein in the supernatant by using a Pierce BCA Protein Assay (Thermo Fisher Scientific). Surface delivery of CB1 was estimated using a sequential labeling protocol. HEK cells expressing SNAP-CB1 were labeled with impermeable SNAP-Cell® 488 (300nM; New England Biolabs) to saturate the surface CB1. They were then incubated with impermeable SNAP-Cell® 647 (100nM; New England Biolabs) for 1 hour in the presence or absence of WIN. The ratio of 647/488 was used to measure the relative amount of new CB1 delivered to the surface.

Fixed-cell Immunofluorescence

After each drug treatment, cells were fixed with 4% paraformaldehyde (P6148; Sigma-Aldrich) for 15 minutes, and processed for immunofluorescence as described recently (Kunselman et al., 2021). After fixation, antibody labeling was performed as follows: Anti-GPP130 (1:1000; provided by Adam Linstedt, Carnegie Mellon University, Pittsburgh, PA). Anti-TGN46 (1:1000; ab50595; abcam). Anti–TGN-38 (1:1000; T9826; Sigma-Aldrich). Anti-GM130 (1:1000; PA5-95727 Thermo Fisher Scientific). Anti-α-tubulin (1:1000; ab185031; abcam). Anti-MAP2 (1:1000; ab5392; abcam). Confocal images were taken on a Nikon Eclipse Ti inverted microscope using a 20x/0.75 NA, 60x/1.49 NA or 100x/1.49 NA objective. The images were acquired with an iXon+ 897 electron-multiplying charge-coupled device camera using Andor IQ software (Andor).

Image analysis protocols

All images were analyzed via ImageJ. We generated custom ImageJ macros for standardized quantification of GPP130 and α-tubulin in HEK cells, TGN-38 in rat striatal neurons (BrainBits, LLC), and GM130 in mouse CB1-KO cortical neurons (Ledent et al., 1999). Briefly, images were

thresholded using a specific value, and the area and the fluorescence of the objects identified above threshold were used for data analysis. The parameters for analysis were kept identical between the control and the experimental conditions.

Statistical Analysis

This exploratory study was designed to address the effect of WIN on membrane trafficking. Sample sizes (number of cells or fields to be analyzed), were determined before the experiment was performed, and the p-values reported are descriptive. All analysis was performed on GraphPad Prism 8, 2019. We determined before viewing the data that each group would be compared to the respective control conditions. Data were analyzed by D'Agostino-Pearson test for normal distribution. For normally distributed data, statistical significance for analysis comparing two experimental conditions was derived from unpaired t-tests, and for three or more conditions by ordinary one-way ANOVAs and multiple comparisons by a Dunnett's test. For data not normally distributed, statistical significance for analysis comparing two experimental conditions was derived from Mann Whitney tests, and for three or more conditions by Kruskal-Wallis test with multiple comparisons by a Dunn's test. All analyses are reported. For each graph, n denotes the number of cells analyzed for each condition for single-cell analyses, or the number of fields analyzed for each condition for conditions that test penetrance of phenotype. Statistical significance is denoted as follows: ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.001$.

Results

WIN Disrupts the Golgi Apparatus.

We first tested the effect of WIN in CB1-expressing HEK293 cells. HEK293 cells stably. expressing SNAP-tagged CB1 were treated with 10μM of either WIN or 2AG for one hour, and labeled with a cell-permeable SNAP label to detect CB1. In control cells, CB1 was present on the cell surface and on intracellular compartments that roughly overlapped with the Golgi apparatus, detected by the cis-Golgi marker GPP130 (Figure 3.1A). This suggests that CB1 could localize to the Golgi in steady state conditions in these cells. This intracellular CB1 localization is consistent with previous reports (Grimsey et al., 2010; Leterrier et al., 2004), as is also observed for other GPCRs such as the δ-opioid receptor in neuronal cells (Shiwarski et al., 2017). This intracellular localization did not change when cells were treated with the endocannabinoid 2AG. In contrast, this intracellular pool of CB1 became more dispersed across the cytoplasm in cells treated with WIN (Figure 3.1A).

This finding raised the question as to whether the dispersed localization induced by WIN was due to specific redistribution of CB1, or whether this was due to general dispersal of the Golgi. To test this, we imaged the behavior of the general Golgi marker GPP130. GPP130 appeared fully dispersed across the cytoplasm upon WIN treatment, compared to the discrete staining seen in untreated cells or cells treated with 2AG (Figure 3.1A). To quantify dispersal of Golgi compartments, we reasoned that dispersal would result in redistribution of fluorescence over a larger area, and, correspondingly, that the fluorescence in each area will be reduced. Therefore, we measured the fraction of total cell area covered by the Golgi. In control and 2AG conditions, the Golgi covered roughly 20% of the cell's total area. In contrast, in WIN-treated cells, the Golgi covered roughly 60% of the cell's total area (Figure 3.1B). We next measured the average

fluorescence of the Golgi marker GPP130 in the three conditions. In WIN-treated cells, the Golgi fluorescence decreased almost three-fold compared to that of control and 2AG-treated cells (Figure 3.1C). This decrease roughly corresponded to the three-fold increase in area. The reciprocal changes in area and fluorescence indicate that the Golgi was dispersed in cells after WIN treatment.

We next developed an automated analysis paradigm using area and fluorescence constraints to detect Golgi dispersion in cells imaged at 20x magnification (Supplemental Figure 3.1; Figure 3.1D). Although this assay was of lower resolution and therefore lower sensitivity, this allowed us to analyze dispersion in a higher throughput and objective manner. Using this assay, we observed that WIN dispersed the Golgi at concentrations ≥ 5μM (Figure 3.1E). Because GPP130 is a cis-Golgi protein, we next used the trans-Golgi network (TGN) marker TGN46 to examine the effect of WIN on the TGN. We found that the TGN46 was also redistributed ≥ 5μM concentrations of WIN treatment (Figure 3.1F).

We performed high resolution confocal imaging to examine if WIN produced a partial effect at lower concentrations, in cells treated with concentrations of WIN ranging from 50nM to 400nM. (Figure 3.1G). Our results show that WIN can fragment the Golgi in concentrations as low as 50nM (Figure 3.1H). The Golgi fragmentation we observed was not an indirect effect of WIN changing cell viability, as a three-hour WIN [10μM] treatment did not affect cell viability as measured by Trypan Blue (Supplemental Figure 3.2).

WIN, but not related alkylindoles, disrupt the Golgi Apparatus rapidly and reversibly.

We next compared the time-course of WIN-mediated Golgi disruption to that of Brefeldin-A (BFA), a fungal metabolite that has been extensively used as a standard to study Golgi collapse and reassembly. HEK293 cells expressing a GPP130-GFP were imaged live every 30 seconds for

10 minutes after treatment with 10μM WIN or 5 μg/mL BFA, as described (Donaldson et al., 1990). WIN caused Golgi disruption within seven minutes of treatment, very similar to BFA (Figure 3.2A). To test if WIN-mediated disruption was reversible, we treated cells with WIN [10μM], washed out the agonist, and imaged GPP130-GFP in the cells for two hours after washout. Over this period, the dispersed Golgi fragments reassembled, and formed a discrete Golgi structure in the cell center between 90 minutes and 120 minutes after washout (Figure 3.2B), comparable to the kinetics of reassembly after BFA (Langhans et al., 2007).

To explore the region of WIN that was involved in Golgi disruption, we tested two other synthetic cannabinoids that shared chemical properties with WIN - the clinically relevant naphthoylindole compound, JWH-018, and the enantiomer of WIN, WIN55,212-3, which differs in chirality at the morpholine group. We imaged HEK293 cells expressing a GPP130-GFP every 30 seconds for 10 minutes after JWH [10μM] and WIN-3 [10μM] treatment. Neither compound caused Golgi disruption, suggesting that the disruption is specific to the stereochemical structure of WIN (Figure 3.2C).

WIN Disrupts Microtubules.

Because Golgi structure and an intact microtubule architecture in the cell are highly interdependent, we investigated whether WIN disrupted the microtubule structure in addition to dispersing the Golgi. In HEK293 cells treated with WIN [10μM] for one hour, the characteristic filament network of the microtubule cytoskeleton was visually disrupted at a similar timepoint as Golgi dispersion (Figure 3.3A). We next attempted to quantify this microtubule disruption using image analysis. The intricacy of microtubule filaments added a substantial degree of difficulty when processing 2D images. Therefore, we enhanced the network by using ImageJ for

standardized tubule segmentation across all sample images, as previously described (Kalkofen et al., 2015). We then measured the fluorescence intensity of the defined network and found a significant decrease in WIN-treated cells compared to control conditions (Figure 3.3B). Our findings are further supported by a strong correlation between Golgi and microtubule disruption. We found that microtubule fluorescence decreased as Golgi fluorescence decreased (Figure 3.3C), which represents a coinciding disruption of both cellular structures after WIN treatment.

WIN Disrupts Golgi in Primary Cultured Neurons.

To test whether WIN could disrupt Golgi in multiple cell types including physiologically relevant neurons, we treated embryonic striatal neurons with WIN and assessed Golgi disruption. Consistent with our results in HEK293 cells, we saw robust dispersal of the Golgi in neurons treated with saturating concentrations, 10μM, of WIN (Figure 3.4A). When Golgi dispersal was quantified by measuring area and fluorescence, the Golgi spread across a larger surface area after WIN [10μM] treatment (Figure 3.4B), and the detected TGN38 fluorescence decreased as the surface area increases (Figure 3.4C). A characteristic feature of the mammalian Golgi is that it is located at the microtubule organizing center in the center of the cell body. Therefore, as an orthogonal and higher sensitivity method to quantify neuronal Golgi disruption and localization, we measured the distribution of fluorescence intensity as a function of the distance from the cell center, by generating radial profiles of antibody fluorescence across a projected 2D field (Figure 3.4D). The workflow for generating radial profile plots is provided in Supplemental Figure 3.3. In control and WIN [1μM] treatment conditions, the Golgi fluorescence showed a clear peak at shorter radial lengths, indicating that Golgi fluorescence was concentrated near the cell center, consistent with an intact Golgi apparatus (Figure 3.4E). When neurons were treated with WIN

[5μM], the Golgi fluorescence was more evenly dispersed across all radial lengths of the neuronal cell body (Figure 3.4E). Interestingly, the fluorescence was overall lower across all radii in WIN-treated neurons, as observed by lower total fluorescence estimated as areas under the curve for radial plot values (Figure 3.4F). This difference suggests that the Golgi is redistributed beyond the neuronal cell body into dendrites, as the estimates of radial profiles are restricted to the neuronal body. These results show that WIN causes Golgi disruption in multiple cell types including physiologically relevant striatal neurons.

As a control, we treated embryonic striatal neurons with 2AG and measured Golgi stability using a similar approach as above. No significant differences in Golgi area (Figure 3.4F) or fluorescence (Figure 3.4G) were detected. Additionally, in both control and 2AG treatment conditions, the Golgi signal is concentrated near the nucleus, as measured through our radial profile quantification approach (Figure 3.4H). These results indicate that WIN, but not 2AG, disrupts the Golgi apparatus in neurons.

Golgi Disruption by WIN is independent of CB1.

In both HEK293 cells and embryonic striatal neurons, WIN treatment at ~ 5μM concentrations resulted in Golgi disruption despite variations in cellular CB1 expression. This raised the possibility that the effects of WIN on the Golgi are independent of CB1 receptor expression. To directly test this, we measured Golgi disruption by WIN in cortical neurons cultured from mice in which CB1 was genetically knocked out (CB1-KO) (Ledent et al., 1999) to determine if CB1 was required for WIN-mediated organelle disruption. Neuronal identity of cultured cells was confirmed using the neuronal marker MAP2. Upon treatment with WIN [10μM], we observed that GM130, a cis-Golgi marker, was dispersed in CB1-KO neurons, as well as neurons cultured

from wild-type littermates (Figure 3.5A). To quantify Golgi disruption in CB1-KO neurons we measured the fraction of total cell area covered by GM130 and the fluorescence intensity of detected antibody signal as in Fig 1. Golgi coverage in the neuronal cell body significantly increased when the Golgi was disrupted (Figure 3.5B), and the fluorescence intensity of detected antibody signal decreased as area increased (Figure 3.5C). Both these were comparable to the effects we observed in HEK293 cells. Golgi dispersal was also evident when radial profile plots were used to quantify dispersion. The radial plots show a fluorescence peak at shorter radial lengths in control neurons, but not neurons treated with WIN [10μM] for 20 minutes or one hour (Figure 3.5D). Together, our results show that WIN can cause Golgi dispersion in multiple cell types, independent of CB1 expression.

Golgi Disruption is PPAR-independent.

Because WIN-mediated Golgi dispersal was independent of the primary receptor CB1, we examined whether WIN acted via its main other known effectors - the nuclear proliferator-activated receptors alpha and gamma (PPARα and PPARγ) (O'Sullivan, 2016). To test if PPAR was required for Golgi disruption, we co-treated HEK293 cells with WIN and either a PPARα inhibitor (GW6471 [10μM) or a PPARγ inhibitor (GW9662 [10μM]) for one hour. Inhibition of neither PPARα or PPARγ blocked Golgi disruption completely when HEK293 cells were treated with WIN, suggesting that PPAR activation was not required for WIN-mediated Golgi disruption. However, GW9662 showed a partial protective effect in comparison to control when HEK293 cells were treated with WIN [5μM] (Figure 3.6A). To assess the penetrance of the phenotype of Golgi disruption, we determined the fraction of fields in which the majority (>50%) of HEK293 cells within the field displayed Golgi disruption after WIN treatment. The Golgi was intact for all

fields of HEK293 cells in control conditions. In contrast, Golgi disruption was observed upon treatment with WIN [5μM] and WIN [10μM] in all cells, irrespective of whether PPAR was inhibited (Figure 3.6B). The quantitation confirmed that GW9662 [10μM] produced a partial protective effect in HEK293 cells treated with WIN [5μM], suggesting that PPARγ, although not required, might play a contributory role in WIN-mediated disruption of the Golgi.

Discussion

In this study, we show that WIN can disrupt the Golgi apparatus across multiple cell types, including primary neurons, with partial disruptions starting at nanomolar concentrations and complete disruption at micromolar concentrations. These general effects of WIN are independent of CB1 expression and are effected through an as-yet undiscovered pathway that could be partially regulated by PPARs.

Our results provide a new context to the use of WIN in experimental and behavioral contexts. The divergent effects observed with WIN have often been considered to be due to the unique ways that it might activate CB1 compared to endocannabinoids. This could certainly be true, and part of WIN's unique effects could be explained by differential activation of CB1. For example, WIN exhibits weakened β-arrestin signaling compared to 2AG (Flores-Otero et al., 2014). However, WIN can also elicit CB1-independent effects. WIN can halt proliferation in oncogenic cells (Emery et al., 2013; Müller et al., 2017; Pellerito et al., 2014) independent of CB1 signaling (Scuderi et al., 2011). Additionally, WIN shows anti-inflammatory and anti-nociceptive properties that have also been identified as CB1-independent (Price et al., 2004). One point of consideration is that WIN caused complete Golgi disruption at micromolar concentrations of WIN. However, even at these concentrations, the effect we observe is still specific to WIN, as similar

concentrations of JWH-018 nor WIN55,212-3 did not cause Golgi disruption. Interestingly, WIN-mediated downregulation of the expression of proteins involved in cell growth and survival was also observed only at micromolar concentrations of WIN (Sreevalsan and Safe, 2013) similar to concentrations that can completely disrupt the Golgi apparatus as we report in this study.

WIN could, however, affect Golgi function even at nanomolar concentrations, even without causing complete collapse of the Golgi. In this context, one clear strength of our quantitative imaging approach is that we can detect small changes in the Golgi architecture, which might be missed by traditional biochemical approaches, with high sensitivity. Because the Golgi apparatus is a processing station for many post-translational modifications, including glycosylation, small changes in the Golgi architecture can cause differences in protein processing without changing trafficking rates overall (Puthenveedu et al., 2006). To the best of our knowledge, the exact concentrations of WIN in different brain regions after administration in mice are not known. In mice injected with 2.5 mg/kg of the synthetic cannabinoid JWH-018 i.p., the serum concentration can reach 250nM (Malyshevskaya et al., 2017). Our results suggest that WIN causes partial fragmentation of the Golgi starting at 50nM (Figure 3.1), even without large changes in protein secretion (Supplemental Figure 3.2B). The disruption of the Golgi and associated changes in processing of select proteins could contribute to the wide-ranging physiological effects of WIN.

Our results suggest that the disruption of the Golgi apparatus is specific to the stereochemistry of WIN. WIN belongs to the aminoalkylindole family of cannabinoids. Some alykylindoles can destabilize microtubules, and promote cell death through direct microtubule binding (Cherry et al., 2016). Moieties linked to the nitrogen of pyrrole rings are the structural determinants of alykylindole-microtubule interactions, where bulkier moieties prevent

microtubule binding (Fung et al., 2017). However, it is unlikely that the effect of WIN on the Golgi is through microtubule disruption by alkylindoles. First, the interplay between microtubules and cytoskeleton is bidirectional, as microtubules are required for a discrete Golgi ribbon to exist at the cell center, and as the Golgi can nucleate microtubules (Wu and Akhmanova, 2017). Second, the dispersal and kinetics of Golgi disruption we observe match BFA, and is faster than reported for microtubule disruption. Third, neither JWH-018 nor WIN55,212-3, which share the alkylindole group, caused Golgi disruption. Rather, the chirality of the morpholine group seems to be important for WIN, as WIN55,212-3 does not disrupt the Golgi. The exact mechanism by which the morpholine group disrupts the Golgi apparatus is not known. It is possible that this group binds to an unknown effector. WIN also contains an oxazine group which has been suggested to have anti-tumor properties, and it is possible that the morpholine group might sterically control the interactions of the oxazine group with its effectors.

Our results indicate that Golgi disruption does not require CB1 activation, as we observed similar effects in HEK293 cells not expressing exogenous CB1, and in primary cells derived from CB1 knockout mice. One potential CB1-independent target of WIN is the PPAR family of nuclear receptors. WIN can activate PPARα and PPARγ (O'Sullivan, 2016) independent of CB1. Both PPARs can regulate lipid metabolism and could contribute to disruption of the Golgi apparatus. However, our results suggest that the effect of WINs cannot be explained fully by activation of PPARs. First, WIN disrupts the Golgi at relatively fast time scales, which makes it unlikely that a transcriptional regulatory mechanism is the primary mediator. Second, blocking PPAR activation was not sufficient to block Golgi disruption (Figure 3.6). PPARγ inhibition caused a partial reduction of WIN-mediated disruption of the Golgi. Interestingly, PPARγ inhibition on its own can down-regulate tubulin expression and inhibit cell growth (Schaefer, 2007), but the timescale

required to observed downstream effects of nuclear receptors is much longer than what is sufficient for observing WIN's effect on cellular structures. WIN could target one of many proteins that have been identified as being critical for maintaining the integrity of the Golgi apparatus. These include trafficking proteins such as the coatomer protein I complex (COPI), which is disrupted by the fungal metabolite BFA (Donaldson et al., 1990; Orci et al., 1991). However, unlike BFA, which preferentially disrupts the stacked Golgi, WIN also disrupts the trans-Golgi network as marked by TGN38 at micromolar concentrations. Future studies that identify a target for WIN will provide us with a better understanding of how WIN regulates Golgi structure, and might provide insights into the regulation of Golgi structure and trafficking in general.

Irrespective of the mechanism, our results highlight a broad effect of WIN on the Golgi apparatus that is likely to confound interpretations of its effects on cannabinoid pharmacology. These effects could contribute to WIN's role as an antiproliferative and anti-inflammatory agent by disrupting a key organelle that is central to membrane trafficking and protein processing. This study therefore generates new considerations to interpret the unique physiological effects of WIN compared to other cannabinoid agonists.

Significance Statement

The synthetic cannabinoid WIN, widely used to investigate the cannabinoid system, also shows unique broader effects at cellular and organismal levels compared to endogenous cannabinoids. Our study shows that WIN can disrupt the Golgi apparatus and the microtubule network in multiple cell types, independent of cannabinoid receptors. These results could explain how WIN reduces surface levels of proteins and could contribute to the unique physiological effects observed with WIN.

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Figures

Figure 3.1. WIN disrupts the Golgi apparatus.

(A) HEK293 cells imaged with confocal microscopy showing SNAP-CB1 and GPP130 labeling after one-hour drug treatment conditions. The Golgi is intact in control and 2AG [10μM] conditions, while GPP130 labeling shows a robust dispersal phenotype for cells in the WIN [10μM] condition. Similarly, we observe a dramatic redistribution of intracellular CB1 after WIN treatment, which we did not observed with 2AG (yellow arrows). Scale bar = 5μm. **(B)** GPP130 labeling covers a larger surface area after WIN treatment as the Golgi is dispersed throughout the cell, **(C)** and the fluorescence intensity of GPP130 correspondingly decreases as the antibody label becomes less concentrated within the defined Golgi area. *(**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; $n \geq 9$).* **(D)** Confocal images shown at 20x magnification used for high-throughput analysis of Golgi disruption. As previously shown, Golgi dispersal is observed after one hour of WIN treatment, and the mask generated from antibody labeling was used to identify Golgi object as defined by pre-determined area and fluorescence constraints. Scale bar = 10μm. Fewer objects were detected in [5μM] and [10μM] treatment conditions for both **(E)** cis-Golgi compartments and **(F)** trans-Golgi compartments. Circles represent biological replicate means (ns, not significant*; $n = 3$ averaged biological replicates*). **(G)** Representative confocal images showing GPP130 labeling and 3D object identification. Using our analysis paradigms, when the Golgi is intact only one 3D object is detected *(white)*, and when the Golgi is fragmented multiple 3D objects are detected. In this example, 2 objects are detected *(white and yellow)*. Scale bar = 2.5μm. **(H)** Golgi fragmentation represented as a percentage of fragmented Golgi detected out of the total number of Golgi, shows that one-hour WIN treatment at nanomolar concentrations causes Golgi fragmentation, but not complete disruption.

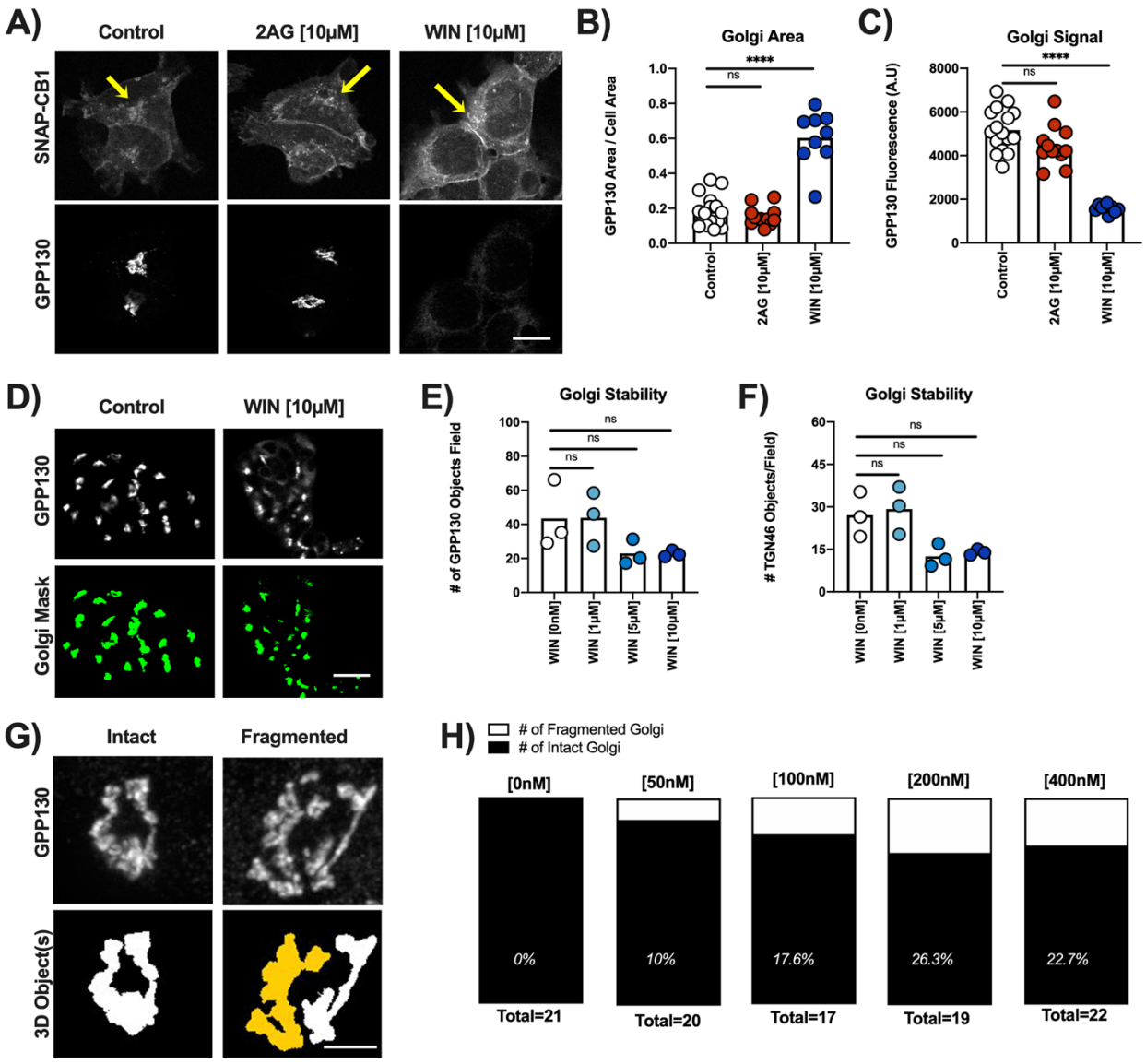

A)
Control
2AG [10μM]
WIN [10μM]
SNAP-CB1
GPP130
B)
Golgi Area
GPP130 Area / Cell Area
****
ns
Control
2AG [10μM]
WIN [10μM]
C)
Golgi Signal
GPP130 Fluorescence (A.U)
****
ns
Control
2AG [10μM]
WIN [10μM]
D)
Control
WIN [10μM]
GPP130
Golgi Mask
E)
Golgi Stability
# of GPP130 Objects Field
ns
WIN [0nM]
WIN [1μM]
WIN [5μM]
WIN [10μM]
F)
Golgi Stability
TGN46 Objects/Field)
ns
WIN [0nM]
WIN [1μM]
WIN [5μM]
WIN [10μM]
G)
Intact
Fragmented
GPP130
3D Object(s)
H)
# of Fragmented Golgi
# of Intact Golgi
[0nM]
[50nM]
[100nM]
[200nM]
[400nM]
0%
10%
17.6%
26.3%
22.7%
Total=21
Total=20
Total=17
Total=19
Total=22

Figure 3.2. WIN-mediated Golgi disruption is comparable to BFA.

(A) Live-cell imaging of HEK293 cells transfected with GPP130-GFP. BFA and WIN disrupt the Golgi at roughly similar time scales. **(B)** Live-cell GPP130-GFP imaging shows that the Golgi partially reassembled within 2 hours of washout after 15 minutes of WIN [10μM] treatment. **(C)** The synthetic cannabinoids JWH [10μM] and WIN-3 [10μM] do not cause Golgi disruption at similar time scales. Scale bar = 2.5μm in all images.

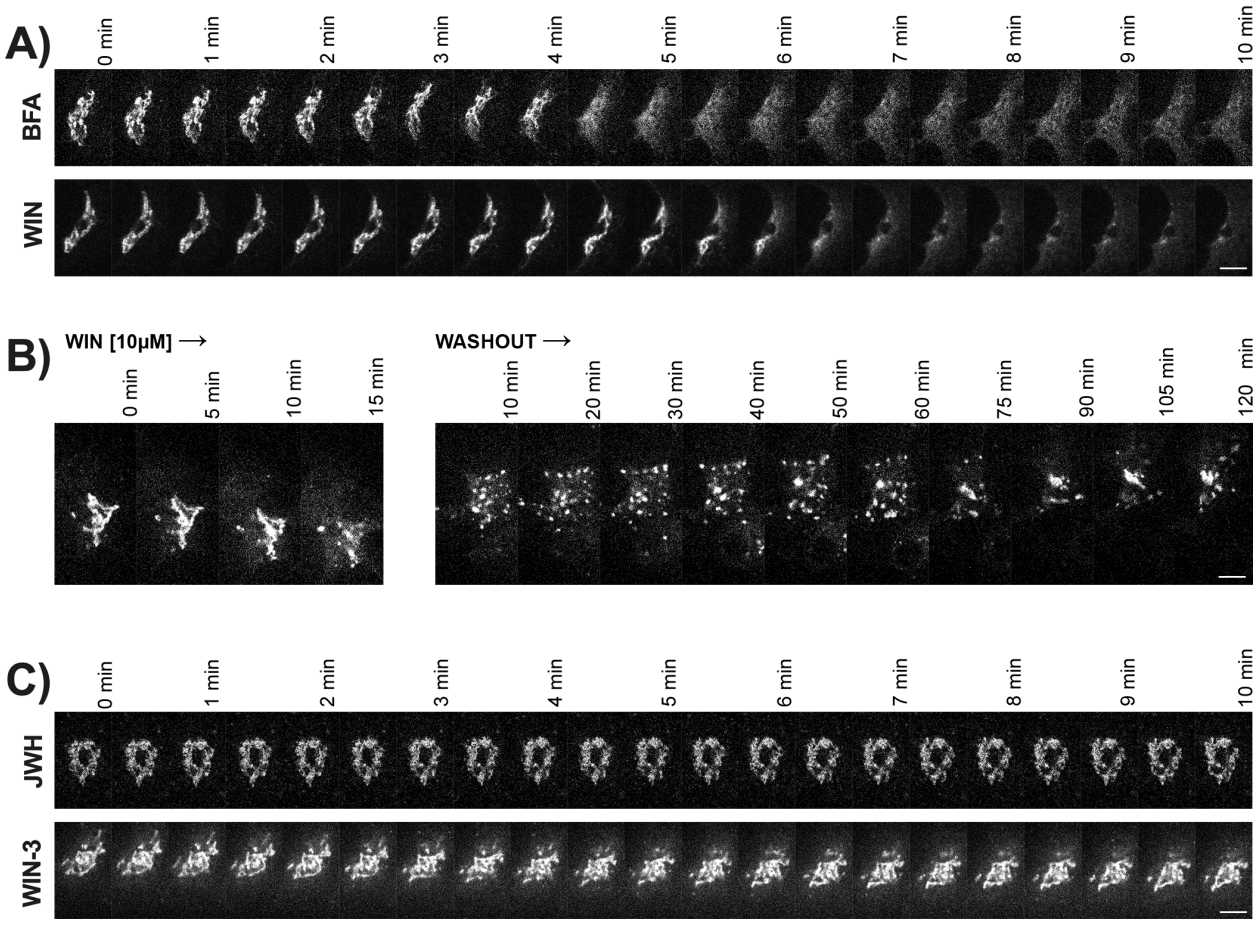


Figure 3.3. WIN disrupts microtubule structure.

HEK293 cells imaged with confocal microscopy showing SNAP-CB1 and α-tubulin labeling after one-hour drug treatment conditions. As shown in Fig. 1, we observe a dramatic redistribution of intracellular SNAP-CB1 after WIN [10μM] treatment. Changes in microtubule organization are also observed, where the tubular network is less defined after WIN [10μM] treatment. α-tubulin labeling was used to generate a microtubule mask to quantify structural differences between treatment conditions. Scale bar = 5μm. **(B)** The fluorescence intensity of α-tubulin labeling is significantly decreased after WIN treatment. *(**, $p < 0.01$; $n = 17$ for control and 20 for WIN).* **(C)** Scatter plot showing the matched fluorescence intensity of α-tubulin and GPP130 values. Two distinct populations are observed, where circles in the control condition (white) have higher microtubule and Golgi fluorescence values than circles in the WIN [10μM] condition (blue).

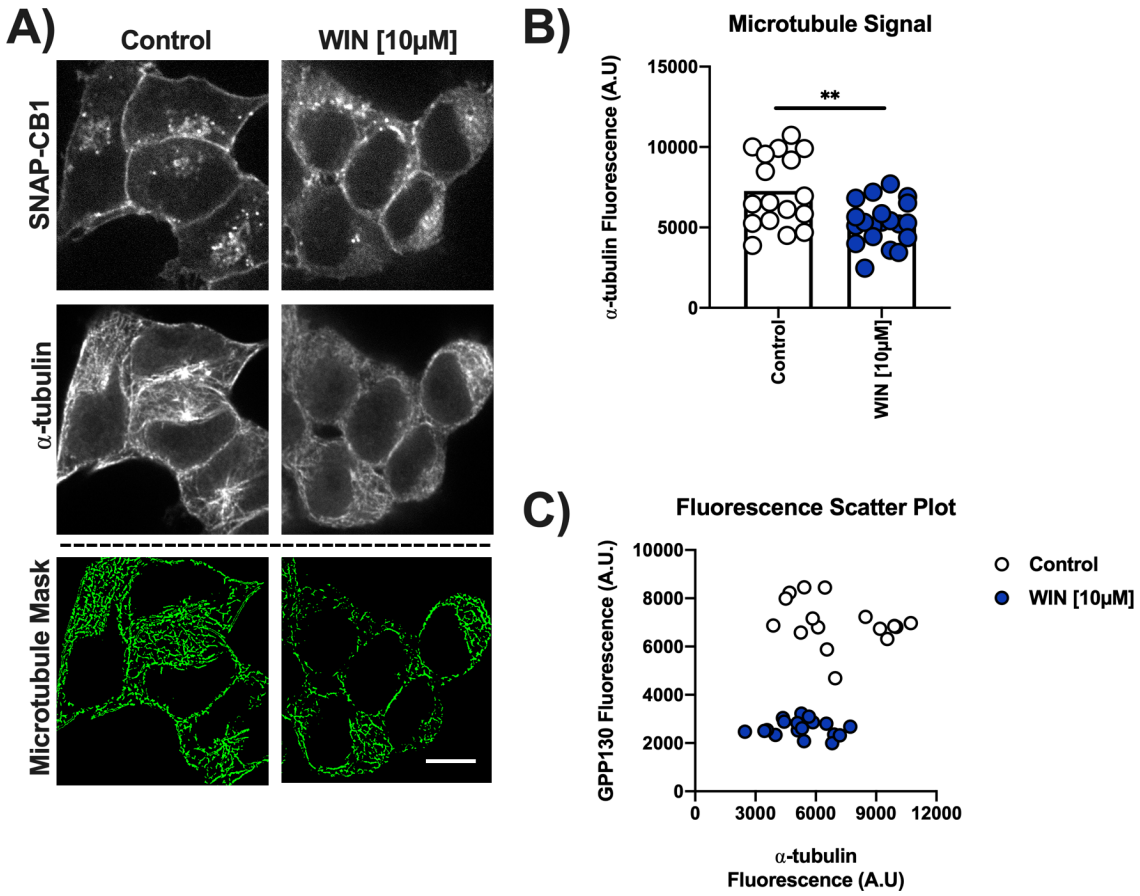


Figure 3.4. WIN disrupts Golgi in primary cultured neurons.

(A) Striatal neurons imaged with confocal microscopy and labeled to detect TGN38 after one-hour control and WIN treatment. As observed in HEK293 cells, Golgi is dispersed in WIN [10µM] treated neurons, but not in control neurons. Scale bar = 2.5µm. By thresholding the TGN38 signal, we quantified differences in area coverage and average fluorescence. **(B)** TGN38 labeling covers a larger surface area after WIN [10µM] treatment, and **(C)** the subsequent fluorescence intensity of TGN38 signal is significantly decreased *(**, $p < 0.01$; ****, $p < 0.0001$; $n \geq 12$)*. **(D)** Schematic depicting Golgi dispersal in neurons and the radial profile quantification approach used to measure TGN38 fluorescence. Fluorescence intensity is concentrated at shorter radial lengths (top panel) when the Golgi is intact, whereas in conditions where the Golgi is disrupted (bottom panel) the fluorescence intensity becomes more evenly distributed across all radial positions. **(E)** Radial plots showing TGN38 fluorescence across radial position at varying concentrations of WIN treatment. Control and WIN [1µM] treated neurons show peaks in fluorescence intensity at shorter radial lengths, while WIN [5µM] treated neurons do not show this robust peak in fluorescence. Error bars represent mean ±95% CI *($n \geq 9$)*. (F) TGN38 labeling does not cover a larger surface area after 2AG treatment, and (G) the subsequent fluorescence intensity of TGN38 signal is not significantly decreased (ns, not significant; *$n \geq 9$)*. **(H)** This is recapitulated with our radial plots where control and 2AG treatment conditions both show peaks in fluorescence intensity at shorter radial lengths. Error bars represent mean ±95% CI *($n \geq 8$)*.

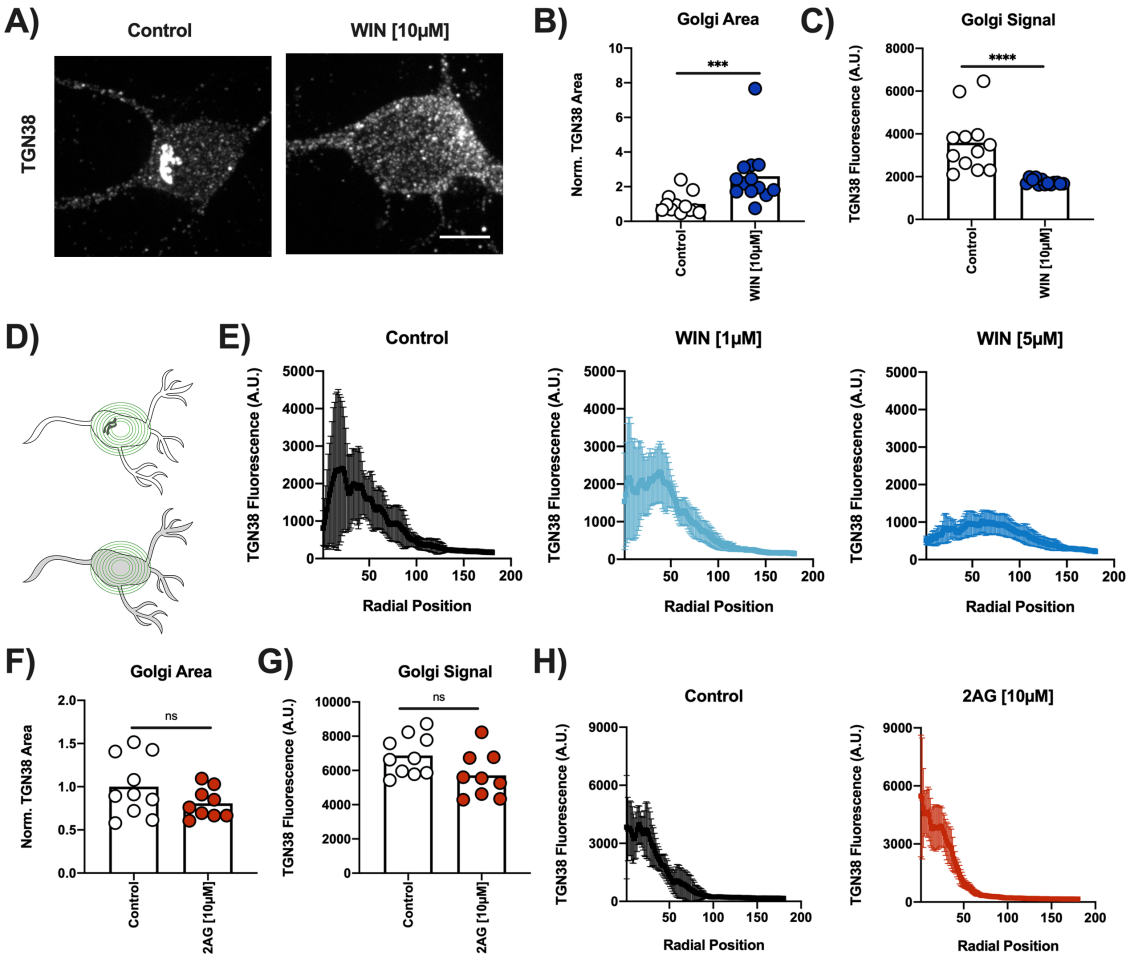


Figure 3.5. WIN-mediated Golgi disruption is CB1-independent.

(A) Cortical neurons of CB1-KO mice and wild-type littermates imaged with confocal microscopy. The Golgi is identified with the cisternal-Golgi antibody, GM130, and neurons are identified with MAP2. As shown in HEK293 cells, the Golgi is intact for neurons in the control group, while GM130 labeling shows a dispersal phenotype for neurons in the WIN [10µM] condition. Scale bar = 2.5µm. **(B)** GM130 labeling covers a larger surface area after WIN treatment, and **(C)** the subsequent fluorescence intensity of GM130 is significantly decreased *(*, $p < 0.05$; $n \geq 9$).* **(D)** Radial plots showing GM130 fluorescence across radial position at varying incubation times of WIN [10µM] treatment. Neurons in the control group show peaks in fluorescence intensity at shorter radial positions, while neurons treated with WIN [10µM] for 20 minutes and one hour do not show this peak. Error bars represent mean ±95% CI ($n \geq 10$)**.**

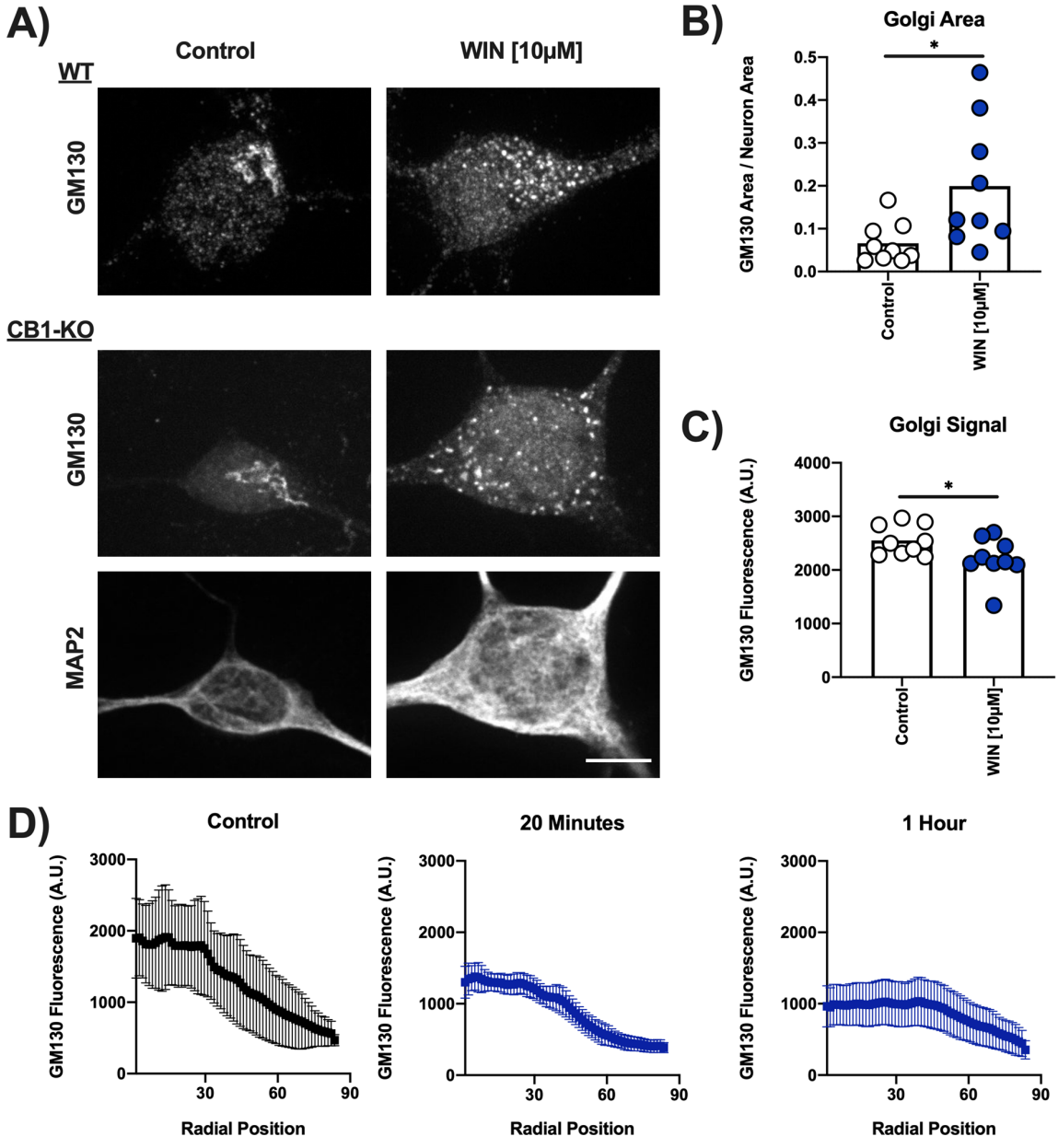
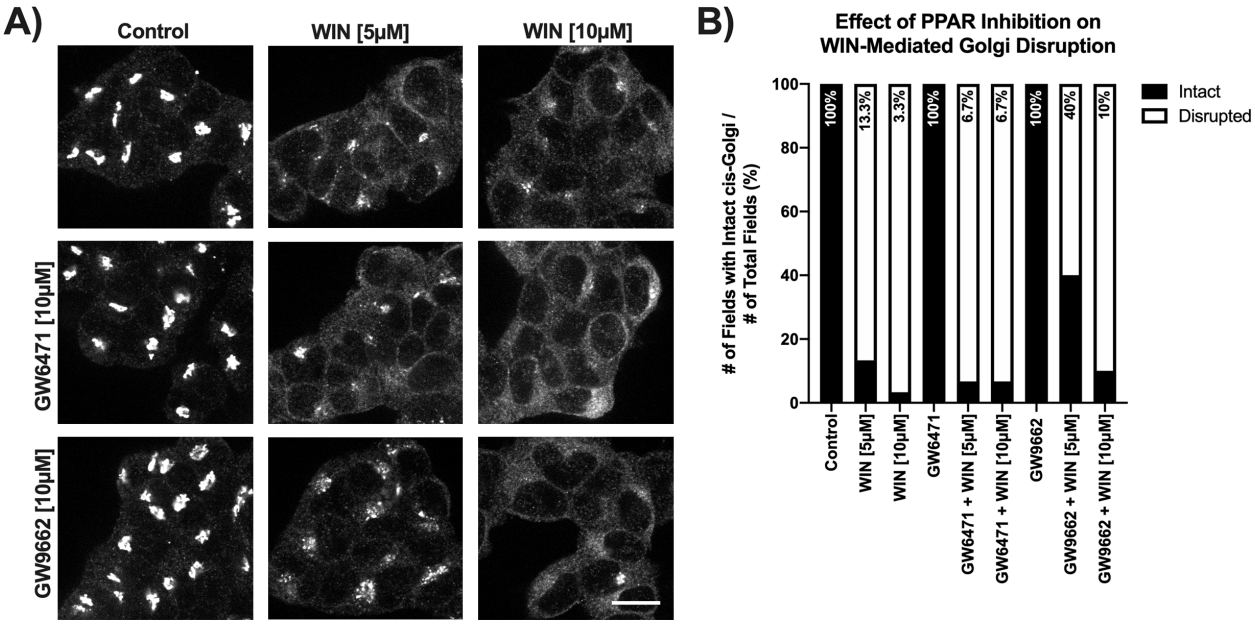


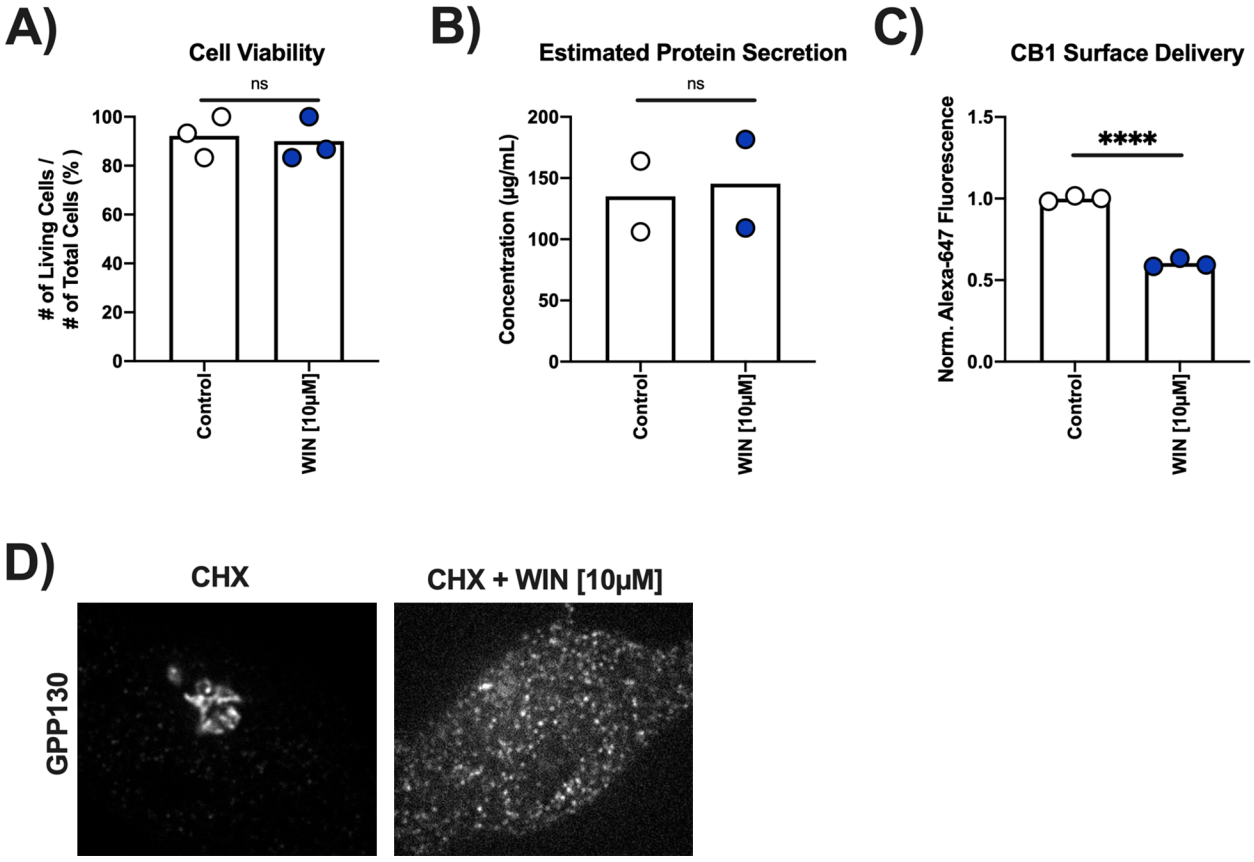
Figure 3.6. WIN-mediated Golgi disruption does not require PPAR activity.

(A) HEK293 cells imaged with confocal microscopy showing GPP130 labeling after one-hour treatment conditions. The Golgi is intact in control, GW6471 [10μM] and GW9662 [10μM] conditions, while GPP130 labeling shows a robust dispersal phenotype after WIN treatment at both [5μM] and [10μM]. Although co-treatment with WIN [5μM] and GW9662 [10μM] collapses the Golgi apparatus, the phenotype is less pronounced than what is observed after treatment with WIN alone. Scale bar = 5μm. **(B)** Stacked bar graph representation of Golgi stability as a percentage of fields exhibiting intact cis-Golgi compartments *(n = 30 for each).*



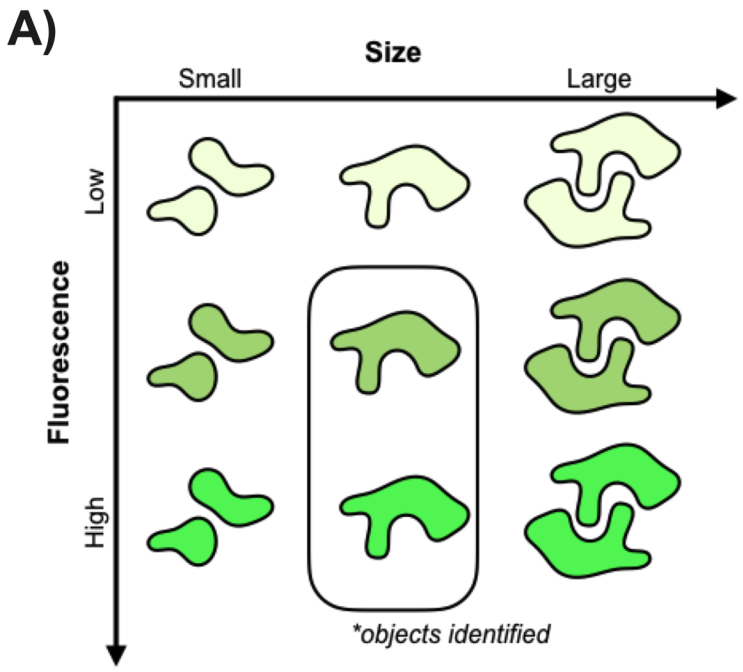
Supplemental Figure 3.1. Area and fluorescence constraints are used for automated detection of Golgi compartments.

The Golgi apparatus will appear as multiple small objects if it becomes fragmented, while multiple intact Golgi compartments may appear as one large object if they are overlapping. This provides an optimal range for detecting singular intact objects based on size. Additionally, a minimum threshold fluorescence for antibody signal can be applied to distinguish detected objects from background noise. Together, these constraints can be used to define Golgi compartments and then quantify the number of objects identified.



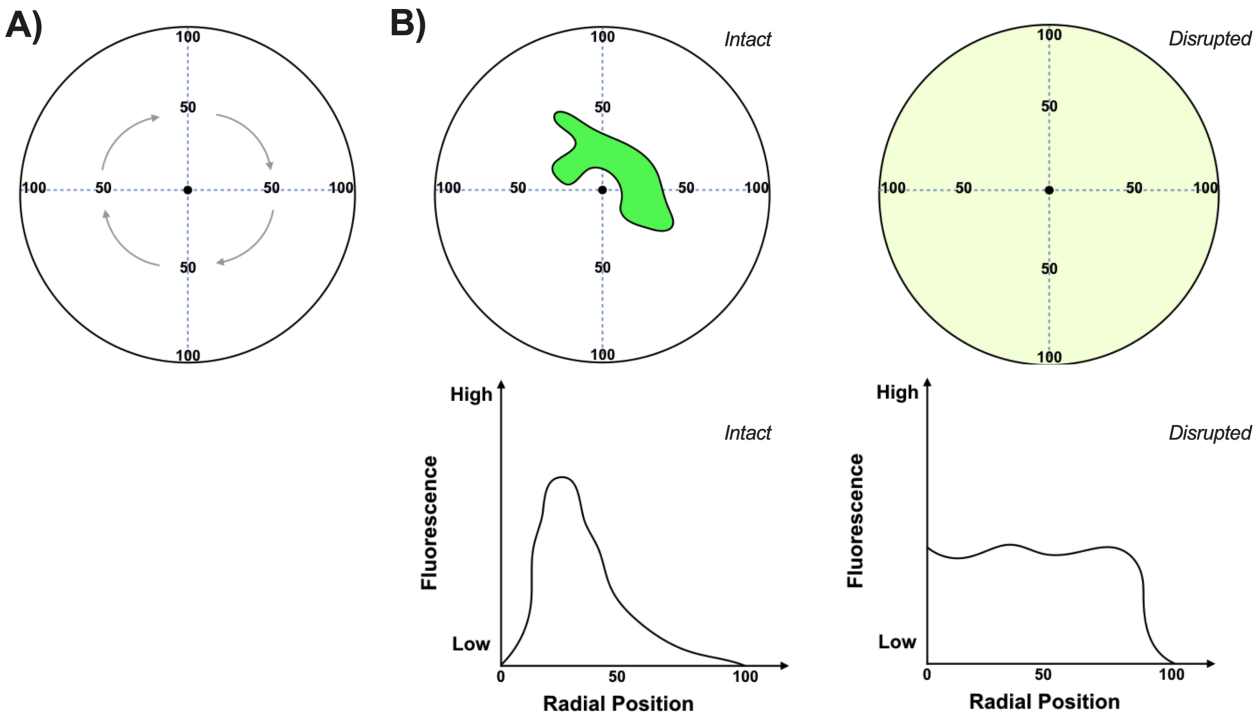
Supplemental Figure 3.2. WIN-mediated Golgi disruption does not cause immediate changes in cellular function.

(A) Bar graph representation of HEK293 cell viability after three-hour treatment with WIN [10μM]. WIN did not significantly increase cell death compared to control conditions. **(B)** Bar graph representation of HEK293 protein secretion after one-hour treatment with WIN [10μM]. WIN did not significantly decrease protein secretion compared to control conditions. **(C)** Bar graph representation of surface delivery of SNAP-CB1 in HEK293 cells over one hour, with and without WIN [10μM]. **(D)** HEK293 cells imaged with confocal microscopy showing GPP130 after two-hour pre-treatment with cycloheximide and one-hour treatment with WIN [10μM]. Cycloheximide did not cause Golgi disruption and did not block WIN-mediated Golgi disruption.



Supplemental Figure 3.3. Golgi dispersal can be quantified through a radial profile analysis.

(A) Radial positions of a circle can be used to measure the distribution of antibody fluorescence throughout the neuronal body. **(B)** An intact Golgi will appear as concentrated antibody signal near the center of the cell, while Golgi dispersal will appear as an even distribution of antibody signal throughout the cell. Plotting antibody fluorescence along radial positions in an XY graph allows for visual representation of intact and dispersed Golgi compartments.



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CHAPTER 4: Discussion

Summary

The work presented in this thesis sought to determine how Cannabinoid Receptor-1 (CB1) trafficking and signaling regulates the specificity of cannabinoid effects. Traditional models of GPCR activation have presumed that receptors only signal from the cell surface, but emerging evidence has demonstrated that GPCRs can signaling from subcellular compartments. Additionally, GPCR signaling is spatially regulated, whereby cell surface activation can initiate a different signaling cascade than subcellular activation. This makes GPCR trafficking an exciting area of cannabinoid research. CB1 exhibits a high degree of subcellular localization in neuronal cells, and the functional significance of subcellular CB1 may underlie the pleiotropic effects of cannabinoid ligands.

Currently, the underlying mechanisms of CB1 function are largely unknown, which makes it difficult to validate specific indications of its therapeutic significance. As discussed in Chapter 1, axonal polarization of CB1 allows for regulation of neurotransmitter release through DSI and DSE. This is primarily regulated by two endogenous cannabinoids, 2-arachidonoylglycerol (2AG) and arachidonoylethanolamine (AEA), both of which modulate synaptic transmission by binding to the cannabinoid receptor-1 (CB1) (Busquets-Garcia et al., 2017). It is speculated that CB1's unique role within the CNS is a neurophysiological component of mood and cognition, and this makes CB1 a probable drug target for interpreting the broad effects of marijuana, as phytocannabinoids are thought to mimic the actions of endogenous cannabinoids within the human

body. Therefore, it is of great interest to understand how CB1 mediates the neurological effects of marijuana.

CB1's ability to modulate synaptic transmission could be regulated by mechanisms of its biosynthetic trafficking. Axonal polarization of CB1 is required for DSI and DSE, therefor CB1 trafficking must be thoroughly investigated to fully understand CB1's function within the CNS. Chapter 2 demonstrates that CB1 is repopulated to the cell surface via biosynthetic trafficking. 2AG upregulates biosynthetic trafficking of CB1 to the cell surface, while AM does not. This represents a potential mechanism by which axonal surfaces are resensitized to acute endocannabinoid stimulation for adequate regulation of neurotransmitter release. It is possible that subcellular populations CB1 may function to replenish surface expression upon agonist-mediated internalization.

CB1 trafficking is regulated by unique interacting proteins. For example, CRIP1a regulates CB1 trafficking from the cell surface to endosomes (Blume et al., 2016), while GASP1 regulates CB1 trafficking from endosomes to lysosomes (Martini et al., 2006). These protein interactions were discovered by investigating CB1 in the context of traditional GPCR trafficking models. However, it is of great interest to determine which proteins are key regulators of CB1's biosynthetic trafficking. Previous work has shown that AP-3 interacts with CB1 to facilitate biosynthetic trafficking to lysosomes (Rozenfeld and Devi, 2008). It is possible that agonist binding disrupts AP-3-CB1 interactions, which would ultimately redirect biosynthetic trafficking of CB1 to the cell surface. Additionally, CB1's conformation upon agoinst binding may drastically change depending on which cannabinoid is bound to the receptor, and this would suggest that cannabinoids differentially regulate the recruitment of accessary proteins to CB1. However, further investigation is required to fully assess this possibility.

WIN 55,212-2 (WIN) has been fundamental in establishing the physiological relevance and therapeutic potential of CB1, and it is now crucial to re-evaluate previous interpretations of WIN-based studies. Since 1992, numerous electrophysiology studies have used WIN to demonstrate CB1's ability to modulate ion channel currents and synaptic release of neurotransmitters within the CNS (Auclair et al., 2000; Katona et al., 2001; Katona et al., 1999; Mackie & Hille, 1992; Shen et al., 1996; Twitchell et al., 1997). Additionally, preclinical research on Alzheimer's and Parkinson's disease have used WIN to investigate the therapeutic potential of CB1 as a drug target (Martín-Moreno et al., 2011; (Price et al., 2009). These examples are by no means comprehensive, as WIN has been used in are over 850 original research articles (Figure 4.1)[1]. However, chapter 3 demonstrates that WIN disrupts the Golgi apparatus independent of CB1, and this significant effect that has gone unnoticed for the past 30 years. This does not dispute the results of previous experiments that have used WIN, but CB1's relevance in those experiments has been largely overinterpreted.

Investigators must utilize cannabinoids that vary by chemical classification, within the same research study, to best interpret the therapeutic significance of the CB1. This classification includes classical, non-classical, aminoalkylindole, and eicosanoid cannabinoids (Pertwee, 2005). However, chemical classification will not fully account for variability in the biological effects of cannabinoids. For example, both THC and CBD are classical cannabinoids, yet they produce drastically different effects (Pertwee, 2005). Additionally, WIN-mediated disruption of the Golgi apparatus is neither universal to synthetic cannabinoids nor the aminoalkylindole class of cannabinoids. Regardless, understanding the chemical specificity of cannabinoid-mediated effects will help elucidate ways in which CB1 could be targeted for specific therapeutic purpose.

In research settings, synthetic cannabinoids will remain instrumental in furthering our understanding of the endocannabinoid system. This is due to the federal regulations associated with studying marijuana and other Schedule I substances. However, similar to phytocannabiniods, synthetic cannabinoids also produce varying biological effects. Additionally, CB1 is implicated in multiple neurophysiological functions (Murillo-Rodriguez et al., 2020), and this will continually present challenges when trying to determine how one cannabinoid may influences a particular neurological state, while another cannabinoid does not.

At present, not enough is known about the cellular and molecular mechanisms of the endocannabinoid system to target it for a specific therapeutic purpose. While Epidiolex is an effective treatment option for severe forms of childhood epilepsy, CBD's therapeutic effects are independent of cannabinoid receptors (Jones et al., 2009). As seen with WIN, it is important to consider the known, and potential unknown, off-targets of cannabinoids when interpreting the biological significance of CB1 activity. It's undeniable that cannabinoids produce a variety of therapeutic effects, but the window of therapeutic implications may not be as vast for the primary receptor target of cannabinoids, CB1.

It is worth noting that cannabinoids target two additional GPCRs; the cannabinoid receptor-2 (CB2) and GPR55 (Pertwee et al., 2010). CB2 is predominantly expressed in the peripheral nervous system where it regulates immune responses (Buckley et al., 2000; Munro et al., 1993), but it also regulates neuroinflammatory responses within in the brain (Ashton & Glass, 2007; Komorowska-Müller & Schmöle, 2020). Modulating neuroinflammation is a desirable approach for treating neurodegenerative disorders, and CB2-selective agonists typically do not have psychoactive properties (Navarro et al., 2016; Walter et al., 2003). Therefore, investigating the pharmacological actions of CB2 may lead to the development of novel therapeutics for CNS

disorders. Alternatively, GPR55 is highly expressed in the CNS (Sawzdargo et al., 1999; Sylantyev et al., 2013). As an orphan GPCR, GPR55 has no known endogenous ligand, but THC acts as its agonist and CBD acts as its antagonist (Lauckner et al., 2008; Ryberg et al., 2007; Whyte et al., 2009). GPR55 is suggested to regulate mood disorders, such as addiction and anxiety (Alavi et al., 2016; Rahimi et al., 2015; Shi et al., 2017; Wróbel et al., 2020), so it is plausible to consider that GPR55, as well as orphan GPCRs that have yet to be identified, could mediate some the effects associated with phytocannabiniods.

Cannabinoids have the potential to become valuable therapeutics for many neurological disorders, though further research is required before specific indications can be determined. Within the CNS, cannabinoids primarily target CB1. Therefore, the cellular and molecular mechanisms that regulate CB1's function must first be established to gain a holistic understanding of its therapeutic potential. This step is essential to advancing cannabinoid pharmacology, because CB1's cellular activity does not mimic what has been established for traditional GPCRs. Strengthening the foundational knowledge of CB1's physiology will delineate cannabinoid effects that are specific to CB1, versus those that are attributed to known, or potentially unknown, off-targets. Most importantly, researchers must be cautious when interpreting the results of their work so that broad generalizations about cannabinoids are not presumed to be directly applicable to CB1.

Limitations

Novel biological mechanisms of CB1 trafficking have been presented in this thesis. However, limitations of the research must be considered when interpreting three key observations. First, constitutive internalization requires clathrin-mediated endocytosis (Fig 2. 1C). Removing

excess SNAP-surface label requires a media washout, but constitutive internalization of SNAP-CB1 can be observed in HEK293 cells within two minutes of SNAP-surface labeling (data not shown). It is possible that the internal puncta observed in control conditions is attributed to CB1 trafficking that occurs during the media washout. This would also explain internal puncta observed in the control condition, as opposed to internal puncta being attributed to clathrin-independent mechanisms of endocytosis. Second, agonist stimulation facilitates delayed internalization of CB1 (Fig 2. 3C). Isoproterenol incubation causes an immediate increase in the number of SNAP-B2 puncta, whereas 2AG incubation causes an increase in SNAP-CB1 puncta after about seven minutes. It is possible that different mechanisms facilitate agonist-mediated internalization of B2 and CB1. If this is true, it is worth comparing agonist-mediated internalization and recycling of CB1 to additional control GPCRs. Finally, 2AG stimulation upregulates repopulation of CB1, but AM stimulation does not (Fig. 2.5B). For all experimental conditions, Alexa-647 fluorescence was quantified as a mean value, and each experimental condition was compared to control to determine statistical significance. However, fluorescence becomes concentrated within internalized puncta after agonist treatment, and this may introduce an unintended variable when comparing internal versus membrane fluorescence. It is possible that AM stimulation causes a significant upregulation of CB1 repopulation that is not detected by this analysis approach.

Expanding Models of GPCR Trafficking

Traditional paradigms of GPCR activation demonstrate two distinct models of trafficking. Upon agonist-mediated internalization, GPCRs are either recycled back to the cell surface for resensitization or they can are trafficked to the lysosome for degradation. However, applying either of these trafficking models to CB1 has remained challenging for two primary reasons. First,

steady-state localization of CB1 is not limited to the cell surface. Subcellular localization of CB1 has been reported in numerous cell types, which makes CB1 unique in comparison to well-studied GPCRs. Additionally, endocannabinoids are lipophilic compounds that easily penetrate membranes, and it is possible that subcellular populations of CB1 are activated independent of receptor internalization. Second, CB1 displays a high degree of constitutive trafficking. Robust CB1 recycling has been reported, consistently, but it has been challenging to explicitly characterize an underlying mechanism of trafficking. This is partially attributed to steady-state lysosomal populations of CB1 observed in both neuronal and non-neuronal cell types. The present challenges suggest that CB1 does not fit into either of these paradigms, exclusively.

The mechanisms that regulate CB1 repopulation remain to be determined. Research presented in this thesis demonstrate a 2AG-mediated upregulation of receptor repopulation, but neither CB1 signaling nor CB1 interacting proteins were assessed is these experiments. An activation-null CB1 mutant that does not bind G-proteins would be the best experimental tool to evaluate the significance of CB1 signaling in receptor repopulation. It would be crucial to determine the rate of repopulation for wild-type and activation-null CB1 receptors in the presence and absence of 2AG. The wild-type and activation-null receptors could show significantly different rates of constitutive repopulation, and this would suggest that CB1 signaling is likely a contributing factor to the mechanism of CB1 repopulation. However, if the wild-type and activation-null receptors show a similar degree of repopulation after 2AG stimulation, then CB1 signaling would not be a contributing factor. In this scenario, specific conformations of CB1 may underlie mechanism of CB1 repopulation, and it is of great interest to determine the significance of CB1-AP-3 interactions. Previous experiments have determined that AP-3 knockdown in Neuro2A cells redirect biosynthetic trafficking of CB1 from lysosomes to the cell surface (Rozenfeld and Devi,

2008). It is reasonable to postulate that 2AG binding to subcellular receptors induces a conformation change of CB1, subsequently disrupting AP-3 interactions. Therefore, AP-3 knockout cells should also be used to measure the repopulation rate of wild-type CB1 in the presence and absence of 2AG stimulation. If 2AG stimulation does not cause an upregulation of CB1 repopulation, then it would suggest that AP-3 transiently interacts with CB1 to regulate biosynthetic trafficking to the cell surface.

Novel experimental approaches are needed to fully elucidate paradigms of GPCR trafficking. Currently, it is well-established that a GPCR's c-terminus mediates a substantial degree of its cellular regulation. Therefore, establishing a host of chimeric GPCRs with substituted c-termini will present an exciting opportunity to further characterize these paradigms. For example, CB1 and B2 chimeras with swapped c-termini can be used to investigate pertinent questions regarding the molecular determinants of GPCR trafficking.

Significance of Science Communication

The trajectory of cannabinoid research has largely been driven by anecdotal reports of their potential health benefits. As a result, cannabinoids garner significant public interest. This interest will continue to grow as more states within the US legalize marijuana for recreational use. As scientists, it will be important to prioritize public education, in the context of cannabinoid research, so that advances in the field are readily communicated to the public.

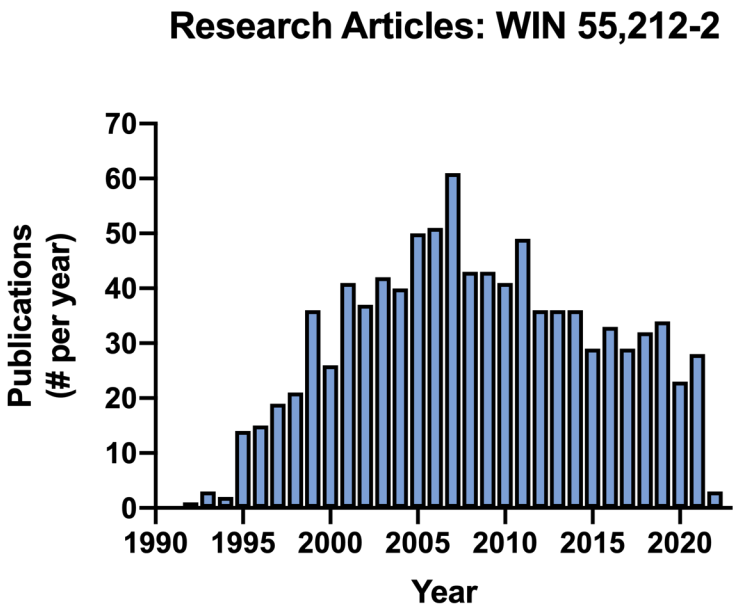
Scientist for Outreach on Addiction Research (SOAR) is an organization at the University of Michigan that I personally helped found and led as President. Through SOAR, I was able to organize events that aimed to make topics of addiction and addiction research more accessible to the general public. I helped organized many events, but the most successful was a panel titled

"*Clearing the Haze: Scientific Discussions on Marijuana and Cannabinoids*", which featured Dr. Allyn Howlett as a panelist. As this field continues to expand, scientists who engage in cannabinoid research have to be equally engaged in communicating their science beyond the walls of academia, because the public is eager to gain a better understanding of how cannabinoids work.

Figures

Figure 4.1. Prevalence of WIN 55,212-2 in cannabinoid research.

The number of publications that used WIN to investigate the endocannabinoid system between the years 1990 and 2022.



Footnotes

[1] Articles: WIN 55,212-2 - www.webofscience.com. https://www.webofscience.com/wos/woscc/summary/9c9a67ec-8207-4a52-a136-d3c74c3bd864-28c2d69c/. Accessed 9 Mar 2022.

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