# Regulation of G protein-coupled receptor trafficking by downstream signaling kinases

by

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### Abstract

Our cells rely on several diverse, extracellular signals to sense and interact with our environment. Many of these signals, such as hormones, neurotransmitters, odorants, taste, and light are transduced by the large family of signaling receptors the G protein-coupled receptors (GPCRs). The role of membrane trafficking of GPCRs in regulating cellular sensitivity to signals has been well described. However, many questions remain about the functional consequences of post-endocytic sorting of GPCRs, the molecular mechanisms that govern this process, and how it is regulated in a physiological context. An emerging paradigm in GPCR biology is that GPCRs undergo endosomal signaling, in addition to cell surface signaling, and the role of GPCR endosomal sorting in regulating this process is unknown.

This thesis investigates how phosphorylation of GPCRs by downstream signaling kinases regulates GPCR endosomal sorting and activity. Chapter 2 shows that hierarchical sorting of GPCRs by signaling kinases switches receptors between endosomal microdomains to control initiation of endosomal G protein signaling. Chapter 3 suggests that the mu-opioid receptor (MOR), the target of endogenous endorphins and clinical opiates, undergoes agonist-selective hierarchical sorting via PKC phosphorylation of the receptor. Chapter 4 of this thesis shows that PKC-dependent sorting of MOR and opioid sensitivity is regulated by substance P signaling in physiologically relevant sensory neurons. Together, the data in this thesis suggest that hierarchical sorting of GPCRs spatially encodes GPCR signaling and that heterologous signaling pathways can regulate GPCR membrane trafficking via receptor phosphorylation.

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## **Chapter 1: Introduction**

### Receptor Biology and G Protein-coupled Receptors (GPCRs)

Understanding how organisms sense their environment and translate extracellular signals into cellular responses that cause physiological changes, complex behavior, and decisions has fascinated scientists for centuries. The use of substances that affect the mind and body long pre-dates the discovery of the molecular targets of drugs. Scientists began to speculate that specific targets for different chemicals existed when they began to learn that hormones, like adrenaline, caused strong and specific effects on the body, for example increased heart rate and blood pressure. Later, adrenaline was shown to only cause these effects on specific tissue and cell types (Ahlquist, 1948). These and observations that different extracts from adrenal glands and nicotine caused muscle contractions, as well as work on antigen interactions with cells, led to some of the first hypotheses on the existence of cell surface receptor molecules (Ahlquist, 1948; Langley, 1905; Maehle, 2004; Silverstein, 1999). However, receptor theory remained controversial for decades, and the receptors themselves were not discovered until over half of a century after the first receptor hypotheses were generated. In the 1970's, Lefkowitz and colleagues created a radiolabeled adrenocorticotropic hormone and detected its binding to adrenal membrane preparations (Hoffman and Lefkowitz, 1990; Mukherjee et al., 1975; Williams and Lefkowitz, 1976). This discovery initiated research focusing on discovery and characterization of receptor targets of hormones and led to the discovery of the largest and most diverse family of ligand-activated receptors, the G protein-coupled receptors (GPCRs).

The first GPCR to be purified was rhodopsin, the retinal receptor that senses light, and other solubilized proteins proposed to be receptors for hormones, like vasopressin and the parathyroid hormone were described in 1975 (Applebury et al., 1974; Krishnan et al., 2012; Lefkowitz, 2013). The epinephrine receptor was the first to be cloned and sequenced and was named the beta-2 adrenergic receptor (B2AR) (Dixon et al., 1986). Since the initial discovery of B2AR, eight hundred GPCRs have been identified that mediate the effects of ligands as diverse as hormones, neurotransmitters, light, odorants, as well as around half of the drugs on the market today (Krishnan et al., 2012; Pierce et al., 2002; Rosenbaum et al., 2009). GPCRs are ligand-activated receptors that transduce a cellular response by coupling to a heterotrimeric G protein to activate downstream signaling cascades.

Several types of pharmacological agents that modulate GPCR-G protein activity exist and have been manipulated and synthesized for clinical use. Agonists bind to GPCRs and stabilize a receptor conformation that activates the G protein. For example, agonists of serotonin and dopamine receptors are used to treat neuropsychiatric diseases and Parkinson's disease (Gainetdinov et al., 2004; Hill, 2006). Antagonists, which compete with the agonist for receptor binding and block the agonist binding site, are also used clinically, such as propanolol, a B2AR antagonist that is used to treat hypertension. Inverse agonists, which bind to the receptor to stabilize an inactive conformation, have also been used as drug targets, such as those that target GABA<sub>b</sub> receptors to modulate their constitutive activity (Agabio and Colombo, 2015; Brown et al., 2015; Hill, 2006). In addition to known GPCR drug targets, several orphan GPCRs

exist that do not have known targets and are likely activated by endogenous molecules, and could be novel drug targets.

Ligand binding of GPCRs induces conformational changes in the receptor that stimulate effector molecules at or near the cell membrane to initiate a downstream signaling cascade. The ligand activated GPCR acts as a guanine nucleotide exchange factor (GEF) for the G protein alpha subunit (Ga) (Bourne et al., 1990; Cassel and Selinger, 1978; Neer and Clapham, 1988). The GTP bound G protein is activated and dissociates from the G<sub>β</sub> subunits (Smrcka, 2008; Wall et al., 1998). Different receptors activate different types of Ga and G $\beta\gamma$  subunits to induce diverse downstream signaling events. For example the prototypical GPCR, the beta-2 adrenergic receptor (B2AR), couples to the Ga stimulatory protein (Gas) and activates the enzyme adenylyl cyclase, which produces the second messenger, cyclic AMP (cAMP) (Cerione et al., 1983, 1984). cAMP production amplifies the signal received by the receptor, and activates downstream signaling kinases, resulting in changes in gene expression, and ultimately a cellular response (Gilman, 1987; Lefkowitz, 2007; Wallukat, 2002). In contrast, other GPCRs, for example the opioid receptors, couple to and activate the Ga inhibitory subunit (Gai) which inhibits adenylyl cyclase, preventing cAMP production (Hsia et al., 1984; Jordan and Devi, 1998; Sharma et al., 1975). GPCR signaling is also transduced by the G $\beta\gamma$  subunits, which often couple to and regulate the activity of ion channels, such as G protein-gated inward rectifier potassium channels (GIRKs) and calcium channels (Huang et al., 1995, 1997; Smrcka, 2008).

After ligand activation of the receptor, post translational modifications are made to the receptor that begin to trigger desensitization of signaling. After activation of the

heterotrimeric G protein and dissociation of the G protein from the GPCR, the receptor is phosphorylated, typically at the C-terminal tail or intracellular loops by G proteincoupled receptor kinases (GRKs), first discovered and described as the beta-2 adrenergic receptor kinase (BARK) (Benovic et al., 1987; Premont and Gainetdinov, 2007; Premont et al., 1995; Wilden et al., 1986). GRK phosphorylation of GPCRs increases affinity for the adapter protein,  $\beta$ -arrestin, named for its ability to "arrest" the G protein signal produced by a ligand by preventing reassociation of the G protein with the receptor (Benovic et al., 1987; Wilden et al., 1986). In addition to inhibiting G protein signaling,  $\beta$ -arrestin serves as a signaling scaffold for GPCRs, initiating a second wave of cell surface signaling for some GPCRs through the Extracellular Signal-regulated Kinases (ERK) pathway (DeWire et al., 2007; Shenoy and Lefkowitz, 2011). β-arrestin also promotes GPCR endocytosis by recruiting endocytic machinery, such as the adapter protein, AP-2, and clathrin, initiating a cascade of events that lead to clathrin coated pit formation, fission of clathrin-coated vesicles by the dynamin GTPase, and trafficking of GPCRs to endosomal compartments where they undergo further sorting and trafficking and are degraded in lysosomes or recycled back to the cell membrane (Drake et al., 2006; Goodman et al., 1996; Hanyaloglu et al., 2008; Zastrow and Kobilka, 1992).

### Regulation of GPCRs by Membrane Trafficking

In addition to regulation of GPCR activity at the cell surface by post-translational modifications, such as phosphorylation and binding of  $\beta$ -arrestin, GPCRs are extensively regulated by membrane trafficking in the endocytic pathway (Bowman and Puthenveedu, 2015; Claing et al., 2002; Drake et al., 2006; Hanyaloglu et al., 2008;

Jean-Alphonse and Hanyaloglu, 2011; Lefkowitz, 2007). Much of the work in GPCR drug development has focused on modifying the receptor itself, through agonist properties, such as efficacy and binding affinity, to alter downstream signaling. However, the role of membrane trafficking of GPCRs in generating or regulating the downstream response to a drug is an area that could potentially lead to the discovery of novel GPCR targeted drugs. There is increasing evidence that implicates defects in receptor membrane trafficking in the development of diseases (Dorsam and Gutkind, 2007; Durieux et al., 2010; Scita and Di Fiore, 2010; West and Hanyaloglu, 2015). Further, different agonists of the same receptor can cause distinct patterns of membrane trafficking of receptors that vary across cell types. For example, morphine does not promote endocytosis of mu-opioid receptors (MORs), the targets of both endogenous endorphins and clinical opiates, in HEK 293 cells, while the synthetic enkephalin DAMGO induces robust internalization of the receptor (Kieth et al., 1996). Interestingly, morphine does promotes endocytosis of MORs in neurites, but not the somas, of cultured nucleus accumbens neurons, and morphine and DAMGO both induce endocytosis of MORs in striatal medium spiny neurons (Haberstock-Debic et al., 2003, 2005). Agonist selective regulation of GPCR trafficking suggests that the endocytic pathway plays an important physiological role in regulating GPCR activity, and a better understanding of the mechanisms of GPCR post-endocytic sorting could potentially aid in the development of novel GPCR targeted therapeutics.

GPCRs can internalize after ligand binding via clathrin-mediated endocytosis, and are subsequently trafficked to endosomes, a step that determines whether they are recycled back to the cell surface or degraded in the lysosome (Fig 1-1) (Hanyaloglu et

al., 2008; Lamb et al., 2001; Marchese et al., 2008; Pierce et al., 2002; Yoburn et al., 2004; Zastrow, 2003; Zastrow and Kobilka, 1992). It is well established that endosomal sorting can increase or decrease the number of receptors on the cell surface via recycling or lysosomal degradation, promoting either resensitization or desensitization to signals, respectively (Alvarez et al., 2002; Gainetdinov et al., 2004; Jean-Alphonse and Hanyaloglu, 2011; Sorkin and Zastrow, 2009). In addition to regulation of surface signaling by membrane trafficking, an emerging paradigm in the GPCR field is that GPCRs undergo G protein signaling from endosomal compartments (Fig 1-1) (Calebiro et al., 2009, 2010; Ferrandon et al., 2009; Irannejad et al., 2013; Jalink and Moolenaar, 2010; Okazaki et al., 2008; Tsvetanova et al., 2015; Werthmann et al., 2012). This suggests that endosomal sorting may play an even more complex role in regulating GPCR activity, perhaps both at the level of insertion and removal of receptors from the cell surface and at the level of endosomal signaling.

The early endosome sorts several diverse transmembrane receptors, including GPCRs, into distinct trafficking pathways. From endosomes, proteins can be sorted to the lysosome for degradation, undergo retrograde trafficking to the Golgi apparatus, recycled by bulk membrane flow, or undergo sequence-dependent, regulated recycling back to the cell membrane (Chia et al., 2013; Dores and Trejo, 2015; Hanyaloglu et al., 2008; Hu et al., 2015; Johannes and Wunder, 2011; Marchese et al., 2008). Many membrane proteins, often nutrient receptors, such as the transferrin receptor (TfR), undergo recycling via bulk membrane flow, independent of requirements for cis sequence elements on the receptor. This bulk, or constitutive recycling, is thought to

occur via geometric sorting, in which narrow membrane tubules protrude from the endosome, creating a high surface area (membrane) to volume (lumen) ratio, inducing



### Fig 1-1. Membrane trafficking of GPCRs regulates sensitivity to cellular signals.

Sequence-dependent recycling of GPCRs recovers sensitivity of cells to extracellular signals by increasing the number of GPCRs at the cell surface. Prolonged desensitization to signals can occur when GPCRs are degraded in the lysosome, decreasing the overall number of functional receptors at the cell surface. In addition to cell membrane G protein signaling, some GPCRs have been shown to undergo endosomal G protein signaling, suggesting a more complex role for membrane trafficking in regulating GPCR activity and sensitivity.

fission and recycling of endosomal tubules (Dunn et al., 1989; Hao and Maxfield, 2000; Mayor et al., 1993). Geometric sorting and recycling of endosomal membrane tubules allows nutrient receptors to rapidly return to the cell surface for subsequent nutrient binding, while their soluble cargoes remain in the endosomal lumen and are degraded in lysosomes as late endosomes mature (Braulke and Bonifacino, 2009; Huotari and Helenius, 2011; Maxfield and McGraw, 2004).

Historically, bulk recycling of membrane proteins from endosomal tubules was thought to be a universally constitutive process, but recent evidence suggests that signaling receptors, like GPCRs, undergo regulated recycling. GPCR regulated recycling requires specific amino acid sequences in the C-terminal tail of the receptor and binding partners, and mutation of GPCR recycling sequences results in lysosomal degradation of these receptors, rather than recycling (Cao et al., 1999; Cong, 2001; Gage et al., 2001; Galet et al., 2004; Gardner et al., 2007; Hanyaloglu et al., 2008; He et al., 2006; Hirakawa et al., 2003; Hu, 2000; Hu et al., 2003; Huang et al., 2004; Li et al., 2002; Tanowitz and Zastrow, 2003; Vargas, 2004). For GPCRs that are targeted to the lysosome for degradation, such as the delta opioid receptor (DOR), lysosomal sorting typically occurs via interactions with ESCRT (Endosomal Sorting Complex Required for Transport) components and sorting of receptors into vesicles that bud into the endosomal lumen and mature and fuse with lysosomes (Babst, 2005; Dores and Trejo, 2015; Marchese et al., 2008; Piper and Katzmann, 2007).

### Mechanisms of GPCR Endosomal Sorting and Post-endocytic Recycling

Post-endocytic recycling of GPCRs has been described for decades in the context of agonist-induced activity and resensitization of cell surface signaling activity (Alvarez et al., 2001, 2002; Galet et al., 2004; Pippig et al., 1995; Tanowitz and Zastrow, 2003; Trejo and Coughlin, 1999; Vargas, 2004; Volpicelli et al., 2002; Zastrow and Kobilka, 1992). Regulated GPCR recycling was first discovered for the B2AR, which requires a specific, C-terminal sequence, DSLL at amino acids 410-413, that interacts with post-synaptic density 95/disc large/zonula occludins-1 (PDZ)-domain containing proteins (Cao et al., 1999; Cong, 2001; Gage et al., 2001; He et al., 2006). Similar recycling sequences that are type I PDZ ligands have been discovered on other GPCRs, such as the beta-1 adrenergic receptor (B1AR) and the kappa opioid receptor (KOR) (Gardner et al., 2007; Hu, 2000; Li et al., 2002). In addition to being required for recycling, these C-terminal sequences are also sufficient to promote recycling when transplanted onto receptors that are typically degraded in the lysosome, such as DOR (Gage, 2004; Gage et al., 2001). The distal C-terminal location of GPCR PDZ ligands is necessary for interactions with PDZ domain containing proteins because the free carboxylate is needed to participate in an ionic bond with the ligand binding groove of the PDZ domain (Karthikeyan et al., 2001; Romero et al., 2011). Further, Sequence comparisons have identified many internal PDZ ligands on GPCRs that might be involved in receptor recycling (Paasche et al., 2005; Romero et al., 2011).

Although several GPCRs are thought to contain conserved PDZ ligand recycling sequences, unique recycling sequences have been discovered on other GPCRs. Unlike the adrenergic receptors, the opioid receptors have diverse trafficking characteristics. There are three opioid receptors, the mu opioid receptor (MOR), the delta opioid

receptor (DOR), and the kappa opioid receptor (KOR). Like the adrenergic receptors, KOR requires a PDZ ligand sequence for post-endocytic recycling (Li et al., 2002). DOR, does not recycle and is degraded in lysosomes following agonist-induced endocytosis (Tanowitz and Zastrow, 2002; Tsao and Zastrow, 2000). The MOR recycles following agonist-induced internalization, but does not require a PDZ-ligand sequence, like KOR and the adrenergic receptors. MOR contains a unique, seven amino-acid recycling sequence in its C-terminal tail, LENLEAE. Mutation of this sequence reroutes MOR to the lysosome following endocytosis, and fusion of this sequence to the Cterminal tail of DOR is sufficient to promote its rapid recycling and prevent lysosomal degradation of DOR (Tanowitz and Zastrow, 2003).

Although MOR's recycling sequence was discovered over a decade ago, the exact mechanism of how this sequence promotes MOR sorting and recycling remains unknown. However, a number of MOR binding partners that regulate MOR trafficking have been found. The actin-binding protein, filamin A, has been shown to interact with the C-terminal tail of MOR, and this interaction is thought to reduce MOR agonist-induced internalization (Onoprishvili et al., 2003). The dendritic spine protein, spinophilin, interacts with MOR in the striatum, and interestingly, knockout of spinophilin reduces sensitivity to morphine-induced analgesia. Further, agonist-induced internalization of MOR is significantly reduced in spinophilin knockout cells (Charlton et al., 2008). Spinophilin interacts with DOR, as well as MOR, and this interaction requires the third intracellular loop, the G protein coupling domain of GPCRs, as well as the first eighteen amino acids of the C-terminal tail, conserved between the two opioid receptors. This interaction enhances ERK signaling through DOR, but not MOR,

suggesting that the interaction with spinophilin may modulate sensitivity of MOR and DOR differentially (Fourla et al., 2012). Spinophilin also interacts with alpha-2 adrenergic receptors and D2 dopamine receptors, also via the third intracellular loops, suggesting that spinophilin may regulate several GPCRs, potentially through G protein coupling (Richman et al., 2001; Smith et al., 1999). The additional requirement of a conserved region of MOR and DOR C-terminal tails for spinophilin binding suggests a potential role in membrane trafficking, while the exact mechanism of regulation remains unknown.

Advances in live cell imaging have allowed for detection of B2AR endosomal sorting dynamics, providing novel insights into the mechanisms of GPCR sequence-dependent recycling. The mechanism of B2AR sequence-dependent recycling involves sorting of B2AR into endosomal microdomains that are biochemically distinct from the endosomal microdomains that undergo constitutive recycling through geometric sorting (Puthenveedu et al., 2010), suggesting that there are diverse populations of endosomal recycling tubules (Fig 1-2). Sequence-dependent recycling endosomal microdomains contain components of the actin cytoskeleton, sorting nexins, and members of the retromer sorting complex, and have been named the actin/sorting nexin/retromer tubular (ASRT) domains (Gallon and Cullen, 2015; Puthenveedu et al., 2010; Temkin et al., 2011). Prior to the discovery of ASRT domains, all endosomal recycling tubules were thought to share the same core trafficking machinery and mechanisms.

The primary role of B2AR's PDZ ligand is thought to be coupling the receptor to the actin cytoskeleton and ASRT domains. Mutating B2AR's PDZ ligand and depleting B2AR PDZ ligand interacting partners, components of the actin cytoskeleton, the

retromer complex, or sorting nexin 27 (SNX27) all prevent B2AR recycling (Lauffer et al., 2010; Puthenveedu et al., 2010; Temkin et al., 2011). Replacing the PDZ domain with an ezrin actin-binding domain is sufficient to cause DOR to recycle, suggesting that



# Figure 1-2. GPCRs undergo sequence-dependent recycling through actin-stabilized (ASRT) tubules.

B2AR's PDZ ligand recycling sequence couples receptors to the actin cytoskeleton for recycling. B2ARs recycle through actin/retromer stabilized tubular domains (ASRT) at the early endosome. This pathway is distinct from constitutive recycling tubules traversed by cargo that recycle through a "bulk", geometric sorting mechanism.

actin interactions are required and sufficient for GPCR recycling (Lauffer et al., 2009).

Since many other GPCRs also have PDZ ligands and bind to PDZ-domain proteins, the

role of the PDZ-linked actin cytoskeleton may be conserved for many GPCRs. The exact mechanism that excludes GPCRs from constitutive recycling tubules is still being actively investigated. Receptor diffusion rates on B2AR endosomes using Fluorescence Recovery After Photobleaching (FRAP), suggest that the mobility of B2AR on endosomes is more restricted than that of constitutively recycling cargo, like the transferrin receptor (TfR) (Puthenveedu et al., 2010). One possibility is that B2AR is unable to diffuse rapidly enough into constitutive recycling tubules that undergo fission quickly. ASRT domains may function to stabilize recycling tubules long enough for B2AR to diffuse into these domains before fission occurs. Understanding how and why GPCRs are restricted to specific populations of recycling tubules will be an interesting future direction in GPCR biology and endosomal sorting.

### Regulation of sequence-dependent recycling by hierarchical endosomal sorting

Recent evidence suggests that intracellular signaling cascades control endosomal sorting of GPCRs. This provides new explanations for how cells might coordinate the diverse cellular responses mediated by different GPCRs at rapid physiological time scales. B2AR recycling is regulated by protein kinase A (PKA), a signaling kinase downstream of B2AR activation (Vistein and Puthenveedu, 2013; Yudowski et al., 2009). Adrenergic signaling through B2AR homologously regulates receptor recycling via PKA phosphorylation at the C-terminal tail at serines 345 and 346. Increased PKA phosphorylation of B2AR, following sustained adrenergic signaling, restricts B2AR to ASRT domains on the endosome. Conversely, non-phosphorylated B2AR enters constitutive recycling tubules (Vistein and Puthenveedu, 2013). This suggests a hierarchical sorting mechanism that allows a cell to fine-tune its responses to extracellular signals. For example, in the case of sustained adrenergic signaling, regulated recycling allows a cell to quickly slow B2AR resensitization by decreasing recycling to decrease the number of functional receptors at the cell surface. Hierarchical control of receptor endosomal sorting provides an additional checkpoint for signaling receptor trafficking. First, receptors are trafficked to the early endosome using the recycling sequence on their C-terminal tail, for example the PDZ ligand on B2AR. After arriving at endosomes, phosphorylation of GPCRs by downstream signaling kinases could allow the cell to alter endosomal sorting and recycling kinetics in response to diverse extracellular signals or cellular states. Whether hierarchical sorting occurs for other GPCRs or is regulated by diverse signaling kinases remains unknown. Further, many cell types express several GPCRs that likely signal concurrently. This raises question; can activation of a signaling kinase by one GPCR regulate the endosomal sorting of another GPCR in the same cell to control its activity?

**Control of Endosomal Signaling by GPCR Endosomal Sorting** The roles of postendocytic trafficking in regulating GPCR signaling have remained controversial. Evidence suggests that after activation, GPCRs are desensitized by phosphorylation and must be dephosphorylated to be resensitized to ligand binding and subsequent signaling. It has been suggested that endocytic trafficking may regulate the phosphorylation state of GPCRs, but changes in the phosphorylation and sensitization states of receptors can also occur at the cell surface (Hanyaloglu et al., 2008; Lefkowitz, 2013; Pierce et al., 2002; Rosenbaum et al., 2009; Williams et al., 2013). As a result, regulated sorting of GPCRs has been thought to primarily regulate receptor resensitization by controlling the kinetics of recycling to the cell surface to increase the

response to extracellular signals (Jean-Alphonse and Hanyaloglu, 2011; Lefkowitz et al., 1998; Zastrow, 2003).

Traditionally, GPCR-G protein signaling has been thought to occur exclusively at the cell membrane, with endosomal signaling occurring mostly through non-G protein mechanisms, such as  $\beta$ -arrestin scaffolding to signaling complexes (DeWire et al., 2007; Shenoy and Lefkowitz, 2005, 2011). However, sustained G protein signaling following GPCR endocytosis has been demonstrated for a number of GPCRs, leading to the hypothesis that G protein signaling can occur at the endosome (Calebiro et al., 2009, 2010; Ferrandon et al., 2009; Kuna et al., 2013; Merriam et al., 2013; Okazaki et al., 2008; Werthmann et al., 2012; West and Hanyaloglu, 2015). The parathyroid hormone receptor (PTHR) continues to signal after receptor endocytosis, and distinct agonists have been shown to induce this type of signaling. Interestingly, PTHRs signaling from endosomes were also shown to associate with  $G\alpha s$ , challenging the traditional view that G protein coupling and signaling occurs primarily at the cell surface (Ferrandon et al., 2009; Okazaki et al., 2008). Internalized thyroid stimulating hormone receptors (TSHR) also couple to Gas and cyclic AMP (cAMP) production following internalization (Werthmann et al., 2012). G protein signaling at endosomes has remained controversial, in part because traditional signaling assays do not discriminate between the cell surface and subcellular compartments as signaling sources. However, recent work using a GFP-tagged nanobody biosensor that recognizes the activated form of the Ga stimulatory protein (Gas), showed that the beta-2 adrenergic receptor (B2AR), can activate Gas at early endosomes (Irannejad et al., 2013). This work showed for the first time spatially resolved endosomal G protein signaling, and suggests that GPCRs

can initiate a second wave of signaling from the endosome. Interestingly, a follow-up study on this work showed that cell surface and endosomal G protein signaling activate distinct transcriptional profiles of downstream gene targets (Tsvetanova and Zastrow, 2014). This raises the interestingly possibility that the spatial origin of G protein signaling in a cell can control the type of response produced. Together, this work suggests that endocytosis and endosomal sorting of GPCRs serves not only to decide whether GPCRs are degraded at the lysosome or recycled to the cell surface, but could regulate diverse intracellular signaling cascades at the early endosome following agonist activation.

Diverse downstream signaling consequences of endosomal signaling and hierarchical sorting of GPCRs by signaling kinases raises several new questions about GPCR biology. For example, does GPCR endosomal sorting regulate endosomal signaling responses, and can hierarchical sorting of GPCRs be regulated in an agonist dependent manner? A role for hierarchical endosomal sorting in controlling initiation of endosomal G protein signaling has yet to be shown. However, agonist selective phosphorylation states have been described for the MOR, raising the possibility that hierarchical sorting could be agonist-dependent. When MORs are activated by high efficacy agonists like fentanyl and the synthetic enkephalin, ([D-ala2, N-MePhe4, Glyol]) (DAMGO), phosphorylation is induced on C-terminal residues, threonine 370 (T370) and serine 375 (S375). However, when MORs are activated by morphine, phosphorylation is induced at serine 375, but not at threonine 370 (Doll et al., 2011). Evidence suggests that threonine 370 is phosphorylated by Protein Kinase-C (PKC), while serine 375 is primarily phosphorylated by GRK2 (Doll et al., 2012), suggesting a

potential level of regulation of MOR by different agonists via phosphorylation by different signaling kinases. Further, substance P (SP), a pain sensitizing neurotransmitter, has also been shown to induce phosphorylation at MOR T370, in addition to PKC activation (Illing et al., 2014; Mann et al., 2015). This heterologous phosphorylation of MOR by SP signaling suggests that GPCR signaling cascades could potentially regulate other GPCRs within the same cell. Whether the downstream signaling kinases of other actively signaling GPCRs within the same cell can heterologously regulate receptor sorting via phosphorylation remains an open question. For example, does SP-induced PKC phosphorylation of MOR at T370 play a role in MOR sorting and activity, and if so what role does this regulation play in MOR-induced analgesia?

### Pain and Analgesia

Pain is essential for survival because it serves as a warning that tissue damage has, or is very likely to occur. Intense pain can be as strong a motivator thirst and hunger. This can change the behavior of the organism to avoid further injury by abandoning a harmful environment. In addition to altering behavior, at the molecular level, pain also initiates a cascade of tissue repair mechanisms. Damaged tissue and inflammatory cells release chemicals that sensitize the organism to pain, giving tissue extra protection while it is damaged (Broom, 2000; Hunt, 2009; Scholz and Woolf, 2002). Although pain has evolved to be a powerful signal that leads to changes in motivation and behavior that protect the organism and initiate healing, negative consequences of pain can also arise (Hunt and Mantyh, 2001; Scholz and Woolf, 2002; Stein et al., 2003). As a result, a method of stopping pain sensation and responses is just as vital to an organism's survival. The endogenous opioids are thought to reduce

nociception in virtually all vertebrates (Bodnar, 2015; Lau and Vaughan, 2014; Stein and Lang, 2009). Opiate drugs are also the most powerful painkillers on the market, and the use of extract from the opium poppy, for both medicine and recreation, long predates the discovery of our endogenous opioid systems. The earliest records of opium use for its euphoric effects date as long ago as 3400 B.C., and it was first used in surgeries around 460 B.C. (Brownstein, 1993).

The active ingredient of opium was isolated in 1806 by chemist Friedrich Wilhelm Adam Sertürner, and it was named morphine after Morpheus, the god of dreams (Brownstein, 1993). Morphine was increasingly injected prior to surgeries and other medical procedures following the invention of the hypodermic needle. Morphine proved to be just as addictive as opium extracts, and heroine was developed in 1898 by Bayer, claiming it was more potent than morphine and less addictive (Brownstein, 1993). Unfortunately, heroine proved to be just addictive, and opiate abuse has continued to rise. Since then, hundreds of new opiate derivatives have been synthesized in hopes of creating an opiate drug with the analgesic potency of opiates, but without the addictive potential, such as Oxy Contin, Hydrocodone, and many other well known prescription opiate drugs. Unfortunately, side effects of opiates such as respiratory depression, constipation, tolerance, and addiction still remain a large problem. However, to this day, opiates still remain the most potent analgesics available. The majority of modifications to opiate drugs have addressed the opioid system at the level of the receptor that transduces the effects of opiate drugs, the mu-opioid receptor (MOR) (Matthes et al., 1996).

### The mu-opioid receptor (MOR)

Opioid receptors were first described based the discovery of distinct binding sites of different opiates in the brain (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). Three separate opioid receptors exist, the mu, delta, and kappa. The three opioid receptors bind preferentially to different types of endogenous opioid peptides (Bodnar, 2015; Gillan et al., 1980). All three opioid receptors and endogenous opioid peptides are expressed in regions of the brain involved in reward, motivation, and addiction, as well as the spinal cord. The majority of opiate drugs preferentially bind MOR over the delta opioid (DOR) or kappa opioid receptors (KOR) (Lutz and Kieffer, 2013; Le Merrer et al., 2009). MOR is responsible for both the analgesic and euphoric effects of opiate drugs like morphine, as well as the development of addictive behaviors, as knockdown of MOR causes a loss of morphine-induced analgesia, reward, and withdrawal behaviors (Matthes et al., 1996).

MOR is expressed in both the central and peripheral nervous systems. In the CNS, opioid receptors are expressed mainly in the limbic system, brain stem, and cortex (Lutz and Kieffer, 2013; Le Merrer et al., 2009). In the PNS, MORs are expressed in both C and Aδ nociceptive, or pain sensing, neurons of the dorsal root ganglia (DRG) (Stein and Lang, 2009; Stein et al., 2003). In response to a noxious stimulus, nociceptors projecting from the DRG transduce action potentials as a result of activation of mechanical or thermal receptors or ion channels in the cell membrane (Hunt and Mantyh, 2001; Scholz and Woolf, 2002). These electrical signals are propagated from the periphery of the sensory neuron, for example in the skin, to the dorsal horn of the spinal cord, through the spinothalamic tract, thalamus, and eventually to the cortex where pain is sensed (Hunt and Mantyh, 2001; Scholz and Woolf, 2001; Scholz and Woolf, 2002).

Both peripheral and central nervous system MORs are thought to contribute to the analgesic and euphoric effects of opiate drugs. Opioid receptors are thought to inhibit pain by a few mechanisms. Opioid receptors are G protein-coupled receptors (GPCRs) and transduce their analgesic effects by coupling primarily to Goi. MOR coupling to the Gai protein inhibits the activity of adenylyl cyclase, preventing cAMP production (Connor and Christie, 1999; Laugwitz et al., 1993; Williams et al., 2001). Opioid receptors induce hyperpolarization of neurons by increasing activity of G proteincoupled inward rectifying potassium channels (GIRKs) through coupling of the  $G\beta y$ subunits with ion channels, lowering membrane potential of the cell to decrease excitability (Henderson, 2015; North and Tonini, 1977; North and Williams, 1985; Pepper and Henderson, 1980; Williams et al., 1982). Additionally, opioid receptors can decrease calcium channel current, reducing release of neurotransmitters that mediate pain sensation, for example SP and calcitonin gene-related peptide (Henderson, 2015; Schroeder et al., 1991; Seward et al., 1991; Williams et al., 2001). Nociceptive and inflammatory signaling cascades can also promote release of opioid peptides from neurons and immune cells, and this process is thought to help reduce pain sensation (Bodnar, 2015; Scholz and Woolf, 2002; Stein and Lang, 2009; Stein et al., 2003).

The pain and opioid systems are clearly physiologically coupled, but many questions remain about the regulation of crosstalk between these systems at the level of the receptors that mediate these systems.. One way to better understand the relationship of pain and analgesia at the molecular level is to uncover the mechanisms of how MOR sensitivity to extracellular signals is regulated. Membrane trafficking of GPCRs, like MOR, has been shown to regulate sensitivity of cellular responses to

signals (Jean-Alphonse and Hanyaloglu, 2011; Sorkin and Zastrow, 2009; Williams et al., 2013). Further, there is increasing evidence that GPCR membrane trafficking can be differentially regulated by different agonists, but the molecular mechanisms of this regulation remains an open question. Hierarchical sorting of GPCRs by signaling kinases is a possible candidate for drug-selective regulation of GPCR trafficking. Additionally, if hierarchical sorting can be controlled by heterologous signals produced by other types of receptors in the same cell remains unknown. In addition to regulation of cell surface GPCR signaling, the recent advance of GPCR endosomal signaling suggests a new potential level of GPCR activity that could be regulated by hierarchical endosomal sorting. This thesis addresses the role of downstream signaling kinases in regulating GPCR activity through endosomal sorting.

### Summary of Thesis Research

Recent evidence suggests that cell membrane and endosomal G protein signaling can generate distinct downstream responses in the cell, but little is known about how G protein signaling is regulated at endosomes. *This raises the question, can endosomal sorting of GPCRs regulate initiation of endosomal signaling?* Using live cell imaging of endosomal G protein activation and by analyzing expression profiles of downstream genes activated by endosomal signaling of the beta-2 adrenergic receptor (B2AR), we asked if B2AR G endosomal G protein signaling is regulated by hierarchical endosomal sorting. *This thesis suggests that hierarchical endosomal sorting of B2AR by the downstream kinase, Protein Kinase A (PKA), regulates initiation of endosomal G protein signaling (Chapter 1).*  The role of B2AR hierarchical endosomal sorting in creating an endosomal signaling bias raises the question, *What is the physiological significance of regulating endosomal signaling by endosomal sorting?* The mu-opioid receptor (MOR) contains two PKC sites in its C-terminal tail, a region of GPCRs that often contains signals that regulate membrane trafficking (Hanyaloglu et al., 2008; Romero et al., 2011; Zastrow, 2003). One site, serine 363 (S363) is constitutively phosphorylated, independent of whether agonist is bound to the receptor (Doll et al., 2011). Interestingly, MOR threonine 370 (T370) is a drug-selective site and is phosphorylated by high efficacy agonists, like fentanyl and DAMGO, but not morphine (Doll et al., 2011). Using live cell imaging, this thesis asks if MOR S363 or T370 regulate endosomal sorting of MOR. *This thesis suggests that MOR S363 and T370 are required to sort MOR into distinct endosomal domains and asks if the drug selective site, T370 can regulate MOR endosomal signaling (Chapter 2).* 

Recent advances in GPCR post-endocytic sorting and endosomal signaling have raised several new and interesting questions about how GPCR sorting regulates receptor function in physiologically relevant cell types. *For example, how do cells integrate the responses of the multiple GPCRs they express?* Using live cell imaging assays, readouts of MOR activity in sensory neurons, and antinociception assays in mice, we asked if MOR trafficking and sensitivity is regulated by pain signaling. *This thesis shows that PKC, downstream of substance P signaling, increases opioid sensitivity by regulating MOR post-endocytic trafficking (Chapter 3).* 

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## Chapter 2: Protein kinase A creates a spatial bias in B2AR endosomal signaling

#### Abstract

Endosomal signaling is an emerging paradigm for G protein-coupled receptors (GPCRs), as it suggests that signals are spatially encoded within a cell. Importantly, whether and how this spatial encoding can be dynamically regulated for a given GPCR is not known. Here we show that endosomal signaling via the prototypical beta-2 adrenergic receptor (B2AR) is regulated by Protein Kinase A (PKA), downstream of adrenergic signaling. B2AR activates Gos on the endosome exclusively in actin/sorting nexin/retromer tubular (ASRT) endosomal microdomains. PKA phosphorylation of B2AR increases the fraction of receptors localized to ASRT domains and biases the downstream transcriptional effects of B2AR to genes controlled by endosomal B2AR signals. Our results show that localization of B2AR to ASRT domains has direct downstream consequences, and that this is a dynamic process that can be leveraged by signaling pathways to tune downstream responses by regulating the spatial origin of G protein signaling.

#### Introduction

G protein-coupled receptors (GPCRs) transduce the majority of signals in our body. (Fredriksson et al., 2003; Pierce et al., 2002; Rosenbaum et al., 2009). GPCRs are activated by ligand binding, inducing a conformational change in the receptor, causing activation of heterotrimeric G proteins, which induce downstream signaling

cascades and a cellular response (Pierce et al., 2002; Rasmussen et al., 2011; Rosenbaum et al., 2009). Ligand activated receptors are desensitized and removed from the cell surface by clathrin-mediated endocytosis (Hanyaloglu et al., 2008; Lamb et al., 2001; Yoburn et al., 2004; Zastrow and Kobilka, 1992). Endocytosed GPCRs are transported to the endosome, from where they are either recycled back to the cell surface or degraded in the lysosome (Bowman et al., 2015; Hanyaloglu et al., 2008; Jean-Alphonse and Hanyaloglu, 2011; Lefkowitz et al., 1998; Magalhaes et al., 2012; Marchese et al., 2008; Vistein and Puthenveedu, 2013; Zastrow and Williams, 2012).

The role of endocytic trafficking of GPCRs has been redefined by recent data that GPCRs can signal not only from the surface, but also from the endosome. While this has been established for a subset of GPCRs (Calebiro et al., 2009; Ferrandon et al., 2009; Okazaki et al., 2008; Werthmann et al., 2012), recent data suggest that endosomal initiation of G protein signaling is a common paradigm even for canonical GPCRs like the beta-2 adrenergic receptor (B2AR). A conformation-specific nanobody biosensor that recognizes the nucleotide-free form of the G $\alpha$  stimulatory protein (G $\alpha$ s) was used recently to show that B2AR can support active exchange of G proteins on endosomal microdomains (Irannejad et al., 2013). Further, cAMP signaling from the cell surface and endosomes induced the expression of distinct gene targets, suggesting that GPCR signaling is spatially encoded (Tsvetanova and Zastrow, 2014).

The emerging paradigm that signals are spatially encoded suggests that a key role of membrane trafficking is to move GPCRs between signaling complexes, for example, from G protein-mediated signaling to arrestin-mediated signaling in clathrin-coated pits at the plasma membrane, and to a spatially discrete G protein-mediated

signaling complex in endosomal microdomains for B2AR. The sorting of receptors into clathrin-coated pits at the cell surface has been well studied for GPCRs, and it is clear that signals can regulate both this sorting and its downstream signaling. In contrast, whether signaling pathways can regulate endosome-based signaling and thereby tune the downstream consequences of GPCR signaling is still not known.

Here we show that endosomal signaling by the prototypical B2AR is controlled by Protein Kinase A (PKA) signaling. Using an engineered conformation-specific nanobody biosensor that detects the actively exchanging form of Gas, we show that endosomal G protein signaling is restricted to actin/sorting nexin/retromer tubular (ASRT) endosomal microdomains, even though the agonist-activated conformation of B2AR was not restricted to these domains. PKA phosphorylation of B2AR on its Cterminal tail increases the proportion of B2AR localized to ASRT microdomains. This localization is required for the transcription of endosome-specific genes downstream of adrenergic signaling. Our results reveal a mechanism for how signaling pathways can dynamically tune cellular responses by controlling the spatial origin of G protein signaling.

#### Results

## PKA activation downstream of adrenergic signaling restricts B2AR to ASRT endosomal microdomains

First, we asked whether B2AR signaling from the endosome was spatially restricted to specific functional domains. As a first step, we directly quantitated dynamic signal-mediated redistribution of B2AR between endosomal microdomains in living cells in real time. To do this, we imaged fluorescently tagged B2AR using live cell confocal fluorescence microscopy in HEK 293 cell stably expressing B2AR. This system has been used extensively in the past, and we and others have confirmed that this accurately reflects the trafficking and signaling of B2AR (Han et al., 2012; Kobilka, 1995; Puthenveedu et al., 2010; Seachrist et al., 2000; Vistein and Puthenveedu, 2013; Yudowski et al., 2009). B2AR localized to the cell surface before addition of the B2AR agonist, isoproterenol (iso) (Fig 2-1A). Incubation with iso, within 5 minutes, caused endocytosis and redistributed receptors to endosomes (Fig 2-1A). Within endosomal membranes, B2AR localized to tubular structures, previously characterized as actin/sorting nexin/retromer tubular (ASRT) domains that mediate sequence-dependent recycling, biochemically distinct from tubules that mediate constitutive recycling (Puthenveedu et al., 2010; Vistein and Puthenveedu, 2013). PKA inhibition increased the percent of B2AR endosomes with greater than one B2AR tubule (Fig 2-1A and S2-1A), consistent with B2AR sorting into both ASRT domains and constitutive tubules ((Puthenveedu et al., 2010; Vistein and Puthenveedu, 2013).

To estimate the proportion of B2AR that localized to ASRT domains, we measured B2AR colocalization with established biochemical markers of these ASRT domains - coronin and sorting nexin 1 (SNX1). Five minutes after iso addition, virtually all B2AR tubular domains colocalized with coronin (Fig 2-1 B, quantitated in 1C) and SNX1 (Fig 2-1D, quantitated in 1E), indicating that B2AR was sorted primarily into ASRT domains. In contrast, ~50% of B2AR tubular domains were devoid of coronin and SNX1 (Fig 2-1B-E) after PKA inhibition. A similar redistribution away from ASRT domains was observed two PKA target sites, S345 and S346, on the C-terminal tail of

B2AR were mutated to alanine (SS>AA), consistent with published data that PKA phosphorylation restricts B2AR to ASRT domains (Vistein and Puthenveedu, 2013). This indicates that inhibiting PKA phosphorylation of B2AR, by either pharmacological PKA inhibition or removal of the phosphorylation sites, dynamically relocates approximately 50% of the B2AR to tubular domains not marked by ASRT components. Previous studies indicate that these domains represent "bulk" recycling tubules that mediate the constitutive (i.e., sequence-independent) recycling of nutrient receptors like the transferrin receptor (Cao et al., 1999; Hanyaloglu and Zastrow, 2007; Maxfield and McGraw, 2004; Puthenveedu et al., 2010); (Vistein and Puthenveedu, 2013). This dynamic and signal-mediated relocation of B2AR provided us with an experimental setup to test the functional relevance of PKA-regulated sorting of B2AR to ASRT domains.



#### Figure 1. PKA activation downstream of adrenergic signaling restricts B2AR to ASRT endosomal microdomains.

A. Example images of B2AR agonist induced endocytosis and PKA inhibition. Before iso, B2ARs localize to the cell surface expression. 5 min after iso, B2AR trafficks to endosomal compartments and is sorted into tubule domains (arrowheads). The PKA inhibitor, KT, was added 5 min after iso. Scale is 5 µm in main image and 2 µm in inset. B. Example images of B2AR and coronin (ASRT marker) with iso and KT. Scale is 2 µm. C. Quantitation of the percent of B2AR tubules per cell that are marked by coronin (ASRT) and not marked by coronin (constitutive) before and after KT. KT sorted B2AR into constitutive tubules. Black bars represent mean and SEM across multiple cells (n iso) = 31 cells; n (KT) = 14 cells. P values were calculated across raw tubule number per cell and are unpaired student's t tests. D. Example images of B2AR and SNX1 (ASRT marker) with iso and KT. E. Quantitation of the percent of B2AR tubules per cell that are marked by SNX1 (ASRT) and not marked by SNX1 (constitutive) before and after PKA inhibition, Black bars represent mean and SEM across multiple cells (n iso only = 11 cells; n PKA inh = 18 cells). P values were calculated across raw tubule number per cell and are unpaired student's t tests. F. Example images of SS>AA agonist induced endocytosis. 10 min after iso, SS>AA is also localized to endosomal compartments and sorted into tubule domains. Scale is 5 µm. G. Example images of B2AR and SS>>AA with coronin (ASRT marker). SS>AA localizes to both ASRT domains (arrowheads) and constitutive tubules (arrows). Scale is 2 µm. H. Quantitation of the percent of B2AR and SS>AAASRT tubules. PKA inhibition sorts B2AR into constitutive tubules. Black bars represent mean and SEM across multiple cells (n B2AR = 13 cells; n SSAA = 14 cells). P values were calculated across 40 raw tubule number per cell and are unpaired student's t tests.

#### B2AR activates Gas exclusively in ASRT endosomal microdomains.

We then asked if B2AR signaling from the endosome could be spatially resolved in the context of dynamic redistribution of B2AR between endosomal microdomains. To do this, we used a GFP-tagged conformational nanobody biosensors that recognize the agonist-activated conformation of B2AR, Nb80 (Irannejad et al., 2013; Westfield et al., 2011). Within 5 min of B2AR stimulation with iso, Nb80 was recruited to B2AR endosomes, consistent with a previous report that B2ARs exist in an active conformation at endosomes (Fig 2-2A) (Irannejad et al., 2013). Nb80 localized to all domains of the endosome that contained B2AR, including both ASRT microdomains and constitutive tubules containing SS>AA (Fig 2-2B, C), suggesting that B2AR is in an active conformation in all regions of the endosome, irrespective of whether B2AR was sorted to sequence-dependent or bulk recycling tubules. As a more direct readout of where Gas was actively exchanging GDP for GTP, we used GFP-tagged Nb37, a nanobody that recognizes the guanine-nucleotide free form of G $\alpha$ s. In contrast to Nb80, Nb37 localized to punctate regions on the endosome at the base of B2AR recycling tubules (Fig 2-2D). When imaged with the ASRT domain marker, cortactin, Nb37 localization was largely restricted to domains containing cortactin (Fig 2-2E, quantitated in F). Interestingly, Nb37 was still localized only to ASRT domains in SS>AA, even when the receptors were partitioned to bulk recycling tubules not marked by ASRT components (Fig 2-2E). Approximately 50% of SS>AA tubules were devoid of both Nb37 and cortactin markers (Fig 2-2F). The distinct localization patterns of the nanobodies that detect active receptor and active Gas indicates that G protein activation is restricted to ASRT domains, even though B2AR may be in active conformation in a

broader region of the endosome. ASRT domains might therefore function as a specific scaffold for recruiting and concentrating G proteins on the endosome.



#### Figure 2. B2AR activates Gos exclusively in ASRT endosomal microdomains.

A. Example image of B2AR, cortactin, and Nb80. Nb80 localizes to the endosome and B2AR tubules. Scale is 5 µm. B. Example images of B2AR and SS>AA with cortactin and Nb80. Nb80 also localizes to endosomes and both ASRT (arrowheads) and constitutive tubules (arrows) containing SS>AA. Scale is 2 µm. C. Quantitation of percent of total tubules per cell that contain either cortactin and Nb80, cortactin or Nb80 only, or neither markers. Nb80 localizes to both ASRT and consitutive tubules with receptor. Bars are mean across multiple cells (WT n = 14; SS>A n = 21 cells). Error bars are standard error of the mean. P values were calculated across raw tubule number per cell and are unpaired student's t tests. D. Example image of B2AR, cortactin, and Nb37. Nb37 localizes exclusively to the base of B2AR tubules (arrowheads), with ASRT domain markers (i.e. cortactin). Scale is 5 µm. E. Example images of B2AR and SS>AA with cortactin and Nb37. Nb37 is recruited exclusively to ASRT domains, not constitutive tubules containing SS>AA (arrows). Scale is 2 µm. F. Quantitation of percent of total tubules per cell that contain either cortactin and Nb37, cortactin or Nb37 only, or neither markers. Nb37 is recruited to ASRT tubules only, containing B2AR or SS>AA, but not SS>AA constitutive tubules. Bars are mean across multiple cells (WT n = 42; SS>AA n = 43 cells). Error bars are standard error of the mean. P values were calculated across raw tubule number per cell and are unpaired student's t tests.

PKA hierarchical sorting of B2AR creates a functional endosomal G protein signaling bias.

Emerging data indicate that production of cAMP, the key second messenger downstream of Gas signaling, at the endosome and the cell surface have distinct downstream effects, as they activate the transcription of different sets of downstream genes (Tsvetanova and Zastrow, 2014). We hypothesized that the extent of B2AR signaling from the endosome could be specifically controlled by dynamic relocation of B2AR to bulk recycling tubules, because Nb37 was not recruited to bulk recycling tubules (Fig 2-2). Because a significant fraction of B2AR recycled via bulk recycling tubules when B2AR phosphorylation by PKA was inhibited (Fig 2-1), this provides a potential mechanism for extracellular signaling cues to regulate adrenergic signaling by controlling the site of signal origination, and therefore bias between surface and endosome-based signals. Therefore, we tested how PKA-mediated relocation of B2AR into ASRT domains or bulk recycling domains changed cAMP activation and gene expression. First, we examined the signaling characteristics of both wild type B2AR and the PKA mutant version, SS>AA, mutant by measuring total iso-induced cAMP production. We detected changes in cAMP levels in live cells using the Epac (Exchange protein directly activated by cAMP) CFP/YFP Förster Resonance Energy Transfer (FRET) sensor. cAMP binds to the Epac FRET sensor, causing Epac to open into an activated conformation, that distances the CFP/YFP fluors, preventing FRET and allowing cAMP production to be detected as a decrease in CFP/YFP FRET (DiPilato et al., 2004; Ponsioen et al., 2004). We expressed the Epac FRET sensor in cells stably expressing either wild type B2AR or SS>AA and measured the CFP/FRET ratio

following addition of iso to stimulate B2ARs. Approximately 2 min after iso, the CFP/FRET ratio increased, indicative of a decrease in CFP/YFP FRET and increase in cAMP production, and this response was sustained over 25 minutes (Fig 2-3A-B). We compared the total cAMP levels in B2AR to SS>AA to compare the response produced by B2AR and SS>AA. Total cAMP increased significantly for both B2AR and SS>AA to a similar degree (Fig 2-3C), suggesting that both versions of the receptor produce a similar amount of cAMP at 20 minutes after iso.

Total cAMP levels would likely increase to a similar degree if Gas was activated at either the cell membrane, after receptor recycling, or from endosomes. Therefore, to ask if PKA-regulated hierarchical sorting of B2AR creates a functional endosomal G protein signaling bias, we used reverse transcription followed by guantitative, real-time PCR to measure the levels of expression of an iso-induced and endocytosis dependent gene target, phosphoenolpyruvate carboxykinase 1 (encoded by PCK1), a known cAMP regulated enzyme (O'Brien et al., 1995; Tsvetanova and Zastrow, 2014). We isolated RNA from HEK 293 cells stably expressing wild type B2AR or SS>AA and measured PCK1 expression, as well as expression of reference genes, beta tubulin (TUBB) and hypoxanthine phosphoribosyl transferase 1 (HPRT1). The raw threshold cycle values (Ct) for TUBB and HPRT1 did not change with iso addition for either B2AR or B2AR SS>AA samples, suggesting that these two genes are stable in the presence of iso and are suitable reference genes to normalize PCK1 expression to (Fig 2-3D-E). PCK1 Cts decreased with iso addition, indicative of increased PCK1 mRNA in iso-treated samples, consistent with an iso-induced increase in PCK1 expression (Fig 2-3F). When PCK1 levels were normalized to TUBB expression in the same mRNA samples, iso induced a

3 fold increase in PCK1 expression (Fig 2-3G). Interestingly, iso did not induce an increase in PCK1 mRNA in B2AR SS>AA samples (Fig 2-3 F-G), suggesting that B2AR SS>AA does not initiate endosomal G protein signaling to the same degree as wild type B2AR. This suggests that sorting of B2AR into ASRT microdomains creates a functional bias toward endosomal G protein signaling, while dephosphorylation of B2AR PKA sites and sorting into constitutive tubules could reverse this effect. Further, we observed a decrease in Cts with iso treatment in HEK 293 cells, while TUBB and HPRT1 reference genes remained stable (Fig S2-2A-C), and PCK1 expression increased by approximately 3 fold with iso (Fig S2-2D), suggesting that endogenous B2ARs also induced PCK1 expression after iso addition. We confirmed that endocytosis is required to increase iso-induced PCK1 expression by pretreating cells with dynamin inhibitor, dynasore, to inhibit endocytosis of B2AR. Treatment with dynasore ablated the isoinduced increase in PCK1 expression, actually resulting in a decrease in PCK1 expression (Fig 2-3J-K), consistent with previous work (Tsvetanova and Zastrow, 2014). Next, we disrupted endosomal ASRT domains by incubating cells with iso and latrunculin A to destabilize actin filaments, and measured PCK1 mRNA levels. Latrunculin A treatment prevented the iso induced increase in PCK1 expression, suggesting that intact ASRT domains are required to induce expression of gene targets downstream of B2AR endosomal G protein signaling (Fig 2-3J-K).



Figure 3. PKA hierarchical sorting of B2AR creates a functional endosomal G protein signaling bias.

A. Example images of the Epac FRET sensor and B2AR. 1-2 min after iso addition, the CFP/FRET ratio increases as cAMP is produced and FRET produced by the CFP/YFP Epac sensor decreases, B2ARs subsequently endocytose. B. Quantitation of CFP/FRET ratio across multiple B2AR cells. Error bars are SEM across multiple cells (n = 10 cells). C. Total cAMP produced at 20 min increased significantly for both B2AR and SS>AA.Data are Tukey box plots of responses of multiple cells (n B2AR = 10 cells; n SS>AA = 9 cells). P values were calculated across the CFP/FRET ratio of multiple cells at 20 minutes after iso and are unpaired student's t tests. D. Paired comparisons of raw threshold cycle of amplification (Ct) detected by SYBR green fluorescence increase over background threshold for TUBB mRNA. Pairs compare iso versus no treatment for both B2AR and SS>AA cells. TUBB Cts did not significantly change with iso addition in either B2AR or SS>AA RNA samples. Each pair represents one experimental replicate (n = 6 for all panels). P values were calculated across each experimental replicate and are unpaired student's t tests. E. HPRT1 Cts did not significantly change with iso addition in either B2AR or SS>AA RNA samples. Each pair represents one experimental replicate (n = 6). P values were calculated across each experimental replicate and are unpaired student's t tests. F. Paired comparisons of Cts for PCK1 mRNA, a known iso-induced and endocytosis-dependent gene product. PCK1 Cts were at earlier cycles with iso, suggesting an increase in PCK1 mRNA as a result of iso addition to B2AR cells. SS>AA PCK1 mRNA levels did not significantly change with iso. P values were calculated across each experimental replicate and are unpaired student's t tests. G. Fold change in expression of PCK1 with iso for B2AR and SS>AA. Black bars represent mean across all experimental replicates, error is SEM across experimental replicates P values were calculated across each experimental replicate and are unpaired student's t tests. H. Paired comparisons of Cts for TUBB mRNA comparing no treatment vs. iso, iso vs. iso + dynasore, and iso + latA (n = 4 for remaining panels). P values were calculated across each experimental replicate and are unpaired student's t tests. I. Paired comparisons of Cts for HPRT1 mRNA comparing no treatment vs. iso, iso vs. iso + dynasore, and iso + latA. HPRT1 J. Paired comparisons of Cts for HPRT1 mRNA comparing no treatment vs. iso, iso vs. iso + dynasore, and iso + latA. P values were calculated across each experimental replicate and are unpaired student's t tests. K. Fold change in expression of PCK1 with iso, iso and dynasore, and iso and latA. Expression was normalized to TUBB for all samples.

#### Discussion

Together, our data suggest that PKA-regulated hierarchical endosomal sorting of B2AR creates an endosomal G protein signaling bias by restricting the receptor to ASRT microdomains. This reveals a potential general mechanism for cells to control what type of response is generated by spatially regulating the subcellular origin of G protein signaling. Dephosphorylation of B2AR at S345-6 and sorting of B2AR into constitutive tubules reverses this endosomal G protein signaling bias (Figure 2-4). Controlling endosomal G protein signaling via hierarchical endosomal sorting could allow a cell to fine tune the type of cellular response that is generated in response to different physiological situations.

In addition to the hierarchical regulation of B2AR endosomal sorting by PKA, B2AR requires a C-terminal PSD95-Dlg1-zo-1 domain (PDZ)-ligand sequence that tethers the receptor to the actin cytoskeleton for recycling (Lauffer et al., 2008; Puthenveedu et al., 2010). Prior to recycling, B2AR is sorted into ASRT domains at the early endosome that control sequence dependent recycling distinct from TfR constitutive recycling, which occurs primarily through "bulk" geometric sorting (Cao et al., 1999; Lauffer et al., 2010; Maxfield and McGraw, 2004; Puthenveedu et al., 2010; Temkin et al., 2011). Previous work from our laboratory showed that adrenergic signaling homologously regulates B2AR endosomal sorting into ASRT domains to control the rate of recycling and resensitization to cell surface adrenergic signaling via Protein Kinase A (PKA) phosphorylation of B2AR at serines 345 and 346 (Vistein and Puthenveedu, 2013). This suggests that downstream signaling cades regulate both the rate of resensitization to cell membrane G protein signaling, via receptor recycling, but

also the spatial initiation of signaling within subcellular compartments by using phosphorylation to control GPCR endosomal sorting into distinct microdomains. Rapid control of receptor endosomal sorting by signaling kinases suggests a general mechanism for cells to generate specific types of signals from discrete locations of the cell in diverse physiological situations. Like the B2AR, Recycling of several GPCRs requires a cytoplasmic, C-terminal sequence (Cao et al., 1999; Gage, 2001; Hanyaloglu and Zastrow, 2007; Hanyaloglu et al., 2008; Tanowitz and Zastrow, 2003), but it is currently unknown if different types of hierarchical endosomal sorting signals exist on other GPCRs to control initiation of G protein signaling. An interesting future direction in GPCR biology will be to ask if different signaling kinases regulate the initiation of endosomal signaling for diverse GPCRs and different G proteins.

Several lines of evidence supporting sustained G protein signaling following endocytosis of receptors exist for different GPCRs, the parathyroid hormone receptor, sphingosine-1 phosphate receptors, thyroid stimulating hormone receptors, and D1 dopamine receptors. This sustained signaling is thought to be initiated from endosomal membranes, and Gas and adenylyl cyclases were localized to subcellular compartments (Calebiro et al., 2009; Ferrandon et al., 2009; Kotowski et al., 2011; Mullershausen et al., 2009). Previous work has shown that the retromer complex, in endosomal ASRT domains, is involved in terminating sustained cAMP responses of internalized parathyroid hormone receptors (PTHRs). Overexpression of retromer subunits was shown to decrease the duration of sustained cAMP signaling of PTHR (Feinstein et al., 2011). This data in conjunction with our result that B2AR only activates Gas from ASRT domains at the endosome suggest that G protein signaling at the

endosome can be both initiated and terminated within ASRT domains. In addition to G protein endosomal signaling, arrestin-mediated endosomal signaling mechanisms have also been demonstrated at the endosome for some GPCRs (Godin and Ferguson, 2012; Shenoy and Lefkowitz, 2011; Wehbi et al., 2013). It is unknown if different types of GPCR signaling are initiated from endosomal domains, or perhaps if biochemically distinct tubules serve as scaffolds for diverse signaling pathways.

Interestingly, we observed recruitment of Nb80, which recognizes the agonistactivated conformation of B2AR, to the entire endosome and tubule domains, while the Nb37, the active Gas sensing nanobody was recruited only to ASRT domains. This opens up a number of questions about the regulation of endosomal G protein signaling. One possibility is that Gas is exclusively anchored to endosomal membranes in ASRT domains, so that agonist-activated endosomal B2ARs can only couple to Gas when they are localized to ASRT domains. Endosomal B2ARs in non-ASRT domains might also couple to other G proteins, rather than Gas. In addition to investigating the role of ASRT domains in the regulation of B2AR G protein coupling, it will also be interesting to test the effect of different adrenergic agonists on signaling-regulated spatial encoding G protein signaling.

Another possibility is that the agonist-activated B2ARs that we observed in non-ASRT domains couple to a G protein other than Gas. Perhaps phosphorylation of GPCRs and hierarchical sorting could serve as a G protein coupling switch at the endosome. In the case of B2AR, PKA has been shown to play a role in switching B2AR coupling to Gai (Daaka et al., 1997), which could support a potential role for signaling kinase-regulated hierarchical endosomal sorting in G protein coupling.

Prolonged G protein signaling from endosomes has been shown to have downstream physiological consequences for some GPCRs. In the case of PTHR, distinct ligands of the receptor that produce sustained signaling regulate trabecular bone volume and cortical bone turnover (Okazaki et al., 2008). Sustained cAMP production via the glucagon-like peptide 1 receptor has been shown to be required for glucose induced insulin secretion (Kuna et al., 2013). In the case of the vasopressin 2 receptor, sustained cAMP production is associated with regulation of renal sodium and water transport (Feinstein et al., 2013). The existence of distinct physiological consequences that are controlled by sustained, endosomal signaling of GPCRs suggests that spatial encoding of G protein signaling may be used by diverse cell types and physiological systems. Understanding the molecular mechanisms and identifying the biochemical machinery involved in regulating endosomal sorting of GPCRs could aid in the development of novel spatial-biased GPCR therapeutic targets.



#### Figure 2-4. PKA-regulated hierarchical sorting of B2AR into ASRT domains creates an endosomal G protein signaling bias.

Following activation of B2AR and adrenergic signaling, PKA phosphorylation of the C-terminal tail of B2AR at serine 345 and 346 restricts the receptor to ASRT endosomal microdomains. B2AR activates Gas exclusively in ASRT microdomains. Preventing PKA phosphorylation of B2AR permits the receptor to sort into constitutive (non-ASRT) tubules, where it does not couple to Gas to produce the endosomal G protein signaling response.

#### **Experimental Procedures**

#### Constructs and Reagents

FLAG-B2AR, FLAG-B2AR S345-6A, cortactin, and sorting nexin-1 constructs have been described previously (Hanyaloglu et al., 2005; Puthenveedu et al., 2010; Vistein and Puthenveedu, 2013). Nb80- and Nb37-GFP plasmid constructs were generously donated to us by Roshanak Irannejad and Mark von Zastrow and described previously (Irannejad et al., 2013). The Epac FRET sensor was purchased from Addgene. HEK293 cells were obtained from ATCC and maintained in DMEM High Glucose (Fisher Scientific) + 10% FBS (Gibco). Cells were transfected with Effectene (QIAGEN). Stable cell lines of FLAG-B2AR and FLAG-B2AR S345-6A were generated with Zeocin (Invitrogen) selection. Cells were passed to 25 mm coverglass 1 day after transfection with actin or retromer markers, or the Epac FRET sensor, and imaged the following day (48 hours post transfection).

Isoproterenol hydrochloride (iso) (Sigma-Aldrich) were prepared as 10 mM stocks in water, and KT-5720 (KT) (Santa Cruz Biotechnology) was prepared as a 10 mM stock in DMSO. Latrunculin A (latA) (Cayman Chemical) was prepared at a concentration of 100  $\mu$ g/mL in ethanol. All drugs were used at a final concentration of 10  $\mu$ M. Dynasore hydrate was prepared fresh for each experiment, dissolved in DMSO, and used at a final concentration of 100  $\mu$ M.

#### Microscope Image Acquisition and Analysis

Confocal images were acquired with an Andor Revolution XD spinning disk system on a Nikon Eclipse Ti inverted microscope equipped with a temperature-, humidity-, and CO2-controlled chamber and a 100x total internal reflection fluorescence

(TIRF) objective (Nikon). Cells were imaged live at 37 °C in Leibovitz's L15 - phenol red (Gibco) with 1% FBS. Solid-state 488-nm, 561-nm, or 647-nm lasers served as light sources. Images were acquired with an iXon+ 897 EMCCD camera. HEK 293 cells stably expressing FLAG-B2AR were labeled at 37 °C, in DMEM high glucose + FBS, with M1 anti-FLAG conjugated to Alexa-647 (Molecular Probes). Cells were imaged 5–15 min after the addition of (iso) and 2–5 min after the addition of KT-5720. Stacks and time-lapse images were collected as TIFF images and analyzed with ImageJ. All fluorescence measurements and quantitations were performed on images acquired directly from the camera without adjustments. Graphing and statistical analyses were performed in Microsoft Excel and Graphpad Prism.

#### Quantitation of Tubule Number with ASRT markers and nanobodies

HEK 293 cells stably expressing FLAG-tagged B2AR or the B2AR PKA mutant (SS>AA) were transiently transfected with coronin-GFP and imaged 48 hours after transfection. Images were acquired 5-10 minutes after iso addition. The total number of B2AR or SS>AA positive tubules per cell that colocalized with coronin-GFP, SNX1-GFP (ASRT markers), Nanobody 37-GFP and Nanobody 80-GFP were manually counted double blind with scrambled file names. Experiments were performed at least three separate times for both B2AR and SS>AA, plotted data show results across all cells from each experimental replicate, and unpaired student's t tests were performed. *cAMP measurements with Epac CFP/YFP FRET sensor* 

iso-induced cAMP production was measured in live cells with the Epac (Exchange protein directly activated by cAMP) CFP/YFP Forster Resonance Energy Transfer (FRET) sensor (DiPilato et al., 2004; Ponsioen et al., 2004). The Epac sensor contains a CFP (405-nm excitation, 470-nm emission) and YFP (515-nm excitation and 530-nm emission). Following cAMP binding to Epac's cAMP-binding domain, a decrease in the FRET signal, CFP excitation at 405-nm and YFP emission at 530-nm is occurs, allowing cAMP production to be measured as a decrease in FRET and is expressed as the CFP/FRET ratio. CFP and YFP images were acquired, in addition to images of labeled FLAG B2AR at 37 °C, every 15 seconds in wide-field, using a 60× 1.49 NA TIRF objective (Nikon). HEK 293 cells stably expressing FLAG-B2AR or the B2AR PKA mutant, SS>AA were transiently transfected with the Epac FRET sensor and imaged 48 hours after transfection. A 5 minute baseline was acquired, and then cells were stimulated with iso. Experiments were performed 2 separate times and 5 XY fields were acquired for each time point, plotted data show results across all cells from each experimental replicate, and unpaired student's t tests were performed. *RNA isolation, Reverse Transcription, and Quantitative, Real-time-PCR.* 

RNA was harvested from HEK 293 cells 2 hours after iso addition, using the RNeasy Plus Mini Kit (Qiagen). Cells were co-treated with latA and iso for 2 hours. Cells were pre-treated with dynasore for 20 minutes, and then treated with iso for 2 hours before RNA isolation. RNA was treated with DNase I, amplification grade (Life Technologies), and reverse transcription was performed with the Superscript First Strand System for RT-PCR (Life Technologies), RNA was amplified using random hexamer primers (Life Technologies). cDNA was then treated with RNase H (Life Technologies) prior to qRT-PCR reactions. Experiments were repeated 2 or 3 separate times with 2 or 3 replicates of each condition within each experiment. Plotted data show results across all replicate wells across all experimental replicates, and unpaired

student's t tests were performed. Primers for gene targets were chosen to amplify

targets 50-100 bp and spanning an exon-exon junction. The following primers were

used to amplify gene targets: TUBB: Forward: 5'-GTGGTACGGAAGGAGGTCGATG-3';

Reverse: 5'-AAGGTGACTGCCATCTTGAGG-3'; HPRT1: Forward: 5'-

GAACCTCTCGGCTTTCCCG-3', Reverse: 5'-CACTAATCACGACGCCAGGG-3';

PCK1: Forward: 5'-CTGCCCAAGATCTTCCATGT-3', Reverse: 5'-

CAGCACCCTGGAGTTCTCTC-3'. TUBB and HPRT1 were chosen as reference

(house-keeping) genes for normalization because they did not change significantly with

iso addition. The SYBR Green Select Master Mix (Life Technologies) was used for qRT-

PCR amplification and detection on a BioRad Cfx RT PCR machine.

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### Figure S2-1. PKA inhibition and B2AR-SS>>AA increase the number of B2AR tubules per endosome.

**A.** Percent of endosomes that contain 0, 1, or greater than 1 tubule before and after PKA inhibition. Bars are mean across 27 cells (ctrl) and 30 cells (PKA inh), error are s.e.m. **B.** Raw data of tubules per cell with or without coronin, data as in Fig 2-1. Black bars show mean and s.e.m. **C.** Raw data of tubules per cell with or without SNX1, data as in Fig 2-1. Black bars show mean and s.e.m. **D.** Percent of endosomes that contain 0, 1, and greater than 1 tubule with B2AR and SS>>AA. Bars are mean across 20 cells (B2AR) and 15 cells (SS>>AA), error are s.e.m. **E.** Raw data of tubules per cell with or without coronin, data as in Fig 2-1. Black bars show mean and s.e.m.



### Figure S2-2. PCK1 expression induced by activation of endogenous B2ARs in HEK 293 cells.

**A.** Paired comparison of TUBB expression before and after iso (n = 4 experiments for all panels). TUBB expression did not change with iso. **B.** Paired comparison of HPRT1 expression before and after iso. HPRT1 expression did not change with iso. **C.** Paired comparison of PCK1 before and after iso. Iso induced a decrease in Ct number of amplification for PCK1. **D.** Fold change in PCK1 expression with iso activation of endogenous B2ARs in HEK 293 cells.

# Chapter 3: Protein kinase C regulates sorting of the mu-opioid receptor into distinct endosomal domains.

#### Abstract

Post-endocytic sorting of signaling receptors determines the long-term consequences of receptor activation and endocytosis. How receptor sorting is regulated by signaling in a physiological setting is a question of broad interest. Here we show that the post-endocytic trafficking of the mu-opioid receptor (MOR), the target of endogenous enkephalins and opiate drugs, is regulated by two phosphorylation sites on its C-terminal tail. Using live-cell imaging assays that resolve receptor sorting and surface delivery of MOR at the level of individual sorting and exocytic events, we show that combinatorial phosphorylation of two sites, S363 and T370, determines the localization of MOR into distinct endosomes. Interestingly, agonist-selective phosphorylation of T370 switches the receptor's recycling between actin-dependent and -independent pathways, and regulates expression of gene targets downstream of endosomal adenylyl cyclase signaling. Together, these results show that agonistdependent phosphorylation states of MOR can induce divergent post-endocytic trafficking routes. Further, our results suggest a more general mechanism for how signaling receptors regulate their post-endocytic trafficking routes to fine tune cellular responses.

#### Introduction

Membrane trafficking plays a crucial role in regulating the functional effects of cell signaling. Small changes in the kinetics of receptor trafficking are sufficient to change

surface receptor levels and cellular sensitivity to signals (Anggono and Huganir, 2012; Hanyaloglu et al., 2008; Jean-Alphonse and Hanyaloglu, 2011; Sorkin and Zastrow, 2009). Several extracellular signals, including drugs, neurotransmitters, and hormones produce cellular responses by activating G protein-coupled receptors (GPCRs) (Gainetdinov et al., 2004; Pierce et al., 2002; Rosenbaum et al., 2009; West and Hanyaloglu, 2015). Ligand binding and activation of GPCRs induces clathrin-mediated endocytosis and removal of receptors from the cell surface (Hanyaloglu et al., 2008; Lamb et al., 2001; Yoburn et al., 2004; Zastrow and Kobilka, 1992). GPCR endocytosis is associated with desensitization of receptor signaling, and prolonged desensitization can occur as result of receptor degradation in lysosomes (Lefkowitz et al., 1998; Magalhaes et al., 2012; Marchese et al., 2008; Zastrow and Williams, 2012). Several GPCRs recycle back to the cell surface, resensitizing the cell to further signals(Bowman et al., 2015a; Hanyaloglu et al., 2008; Vistein and Puthenveedu, 2013).

GPCR recycling is a tightly regulated process, in contrast to constitutive recycling of lipids and nutrient receptors through "bulk" geometric sorting, such as the transferrin receptor (TfR) (Cao et al., 1999; Hanyaloglu and Zastrow, 2007; Maxfield and McGraw, 2004; Puthenveedu et al., 2010). Recycling of several GPCRs requires a cytoplasmic, C-terminal sequence, and mutation of this sequence reroutes receptors to the lysosome for degradation (Cao et al., 1999; Gage, 2001; Hanyaloglu and Zastrow, 2007; Hanyaloglu et al., 2008; Tanowitz and Zastrow, 2003b). A mechanism for sequence dependent recycling has been identified for the beta-2 adrenergic receptor (B2AR). B2AR requires a C-terminal PSD95-Dlg1-zo-1 domain (PDZ)-ligand sequence that tethers the receptor to the actin cytoskeleton for recycling (Lauffer et al., 2008;

Puthenveedu et al., 2010). Prior to recycling, B2AR is sorted into actin/sorting nexin/retromer tubular (ASRT) domains at the early endosome that control sequence dependent recycling distinct from TfR constitutive recycling (Lauffer et al., 2010; Puthenveedu et al., 2010; Temkin et al., 2011). Interestingly, recent evidence suggests that adrenergic signaling through B2AR activation homologously controls B2AR endosomal sorting to regulate resensitization to adrenergic signaling. Phosphorylation of B2AR by protein kinase A (PKA), downstream of B2AR activation, restricts B2AR to ASRT endosomal microdomains (Vistein and Puthenveedu, 2013). Inhibition of phosphorylation at two C-terminal PKA sites on B2AR, serine 345 and 346, allows the receptor to enter actin-independent constitutive recycling tubules at the endosome, resulting in faster B2AR recycling and increased resensitization to adrenergic signaling (Vistein and Puthenveedu, 2013).

While the mechanism of sequence-dependent recycling has been well studied for the prototypical GPCR, the B2AR, it remains unknown if the same mechanism of sequence-dependent recycling through endosomal ASRT domains and kinaseregulated hierarchical sorting is conserved across the GPCR-ome. Several GPCRs require a PDZ ligand sequence for recycling, like B2AR (Hanyaloglu et al., 2008), suggesting that an ASRT domain-dependent recycling mechanism could be a universal endosomal sorting mechanism for GPCRs. Interestingly, the mu-opioid receptor (MOR), the target of endogenous endorphins and addictive opiate drugs, contains a unique Cterminal recycling sequence, LENLEAE, that is both required and sufficient for recycling, and mutation of this sequence causes MOR to be degraded in lysosomes (Tanowitz and Zastrow, 2003a). It is unknown if MOR recycling requires endosomal ASRT

domains or if MOR endosomal sorting is subject to hierarchical regulation by downstream signaling kinases, like the B2AR.

Here we show that Protein Kinase C (PKC) switches MOR between actin dependent and independent recycling pathways. This regulation occurred via phosphorylation of MOR at serine 363 (S363) and threonine 370 (T370). MOR S363 and T370 regulate MOR by sorting the receptor into distinct endosomal domains. Interestingly, while MOR S363 is phosphorylated independent of agonist binding, T370 is phosphorylated when the receptor is activated by the synthetic enkephalin, DAMGO, but not morphine (Doll et al., 2011). Preventing phosphorylation at the drug-selective site, T370, resulted in upregulation of gene products downstream of endosomal G protein signaling and adenylyl cyclase activation (Tsvetanova and Zastrow, 2014), Together, these results suggest that MOR activity is regulated by drug selective phosphorylation at the level of endosomal sorting.

#### Results

#### MOR PKC sites, S363 and T370 regulate actin-dependent recycling of MOR

To determine if MOR is sorted through endosomal ASRT domains, we first asked if MOR recycling requires actin. To visualize and quantitate individual MOR recycling events, we imaged MOR N-terminally tagged with a pH-sensitive green fluorescent protein (SpH-MOR) in HEK 293 cells (Miesenböck et al., 1998; Yudowski et al., 2006). SpH fluorescence is quenched in acidic endosomal compartments and dequenced as receptors recycle back to the cell surface, allowing for detection of rapid GPCR recycling events (Bowman et al., 2015b; Puthenveedu et al., 2010; Yu et al., 2010; Yudowski et al., 2006). Live cell imaging using total internal reflection fluorescence
microscopy (TIR-FM) allowed for detection of MOR exocytic events as transient bursts of fluorescence (Fig 3-1 A). Exocytic bursts showed localized peaks of maximum fluorescence intensity, diffusing over time as receptor vesicles fuse with the cell surface, shown by surface plot of intensity (Fig 3-1 B). These events were distinct from endocytic clusters in maximum intensity profile and duration, which persist several frames longer than exocytic events (Fig 3-1 C).

We asked if MOR recycling is dependent on actin by incubating SpH-MOR expressing cells with latrunculin A (latA) to destabilize actin filaments after activation of MOR with the synthetic enkephalin, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) to induce endocytosis and recycling. We determined the percent change in recycling events after latA addition by normalizing each cell to its initial exocytic rate with DAMGO before latA. After latA addition, MOR recycling events decreased by half (Fig 3-1 D-E), suggesting that MOR recycling is partially dependent on actin machinery. Interestingly, B2AR recycling is almost entirely dependent on actin in the same recycling assay (Puthenveedu et al., 2010; Vistein and Puthenveedu, 2013). Ligand-activation of MOR activates PKC via the PLC pathway through MOR coupling to  $G\beta\gamma$  subunits(Henderson, 2015; Smrcka, 2008; Williams et al., 2013), and we have previously shown that MOR recycling and resensitization is regulated by PKC, downstream of signaling through the Ga<sub>a</sub>-coupled GPCR, the neurokinin-1 receptor (NK1R)(Bowman et al., 2015a). Therefore, we asked if PKC regulates MOR's dependence on actin machinery for recycling through PKC phosphorylation of MOR at S363 and T370 by generating single alanine mutants at each site to prevent phosphorylation. Interestingly, when S363 was mutated to alanine (MOR-S363A), MOR recycling was no longer actin dependent (Fig.

3-1 D-E). When T370 was mutated to alanine (MOR-T370A), MOR recycling was slightly more dependent on actin than the wild type receptor (Fig 3-1 D-E). To globally quantitate MOR recycling across several cells, we imaged multiple XY fields of several cells expressing SpH-MOR and measured surface fluorescence levels to quantitate recycling. Upon DAMGO addition, whole cell SpH-MOR fluorescence decreases as receptors traffic to acidic endosomes, and increases as receptors recycle (Fig 3-1 F-G). After a 20 minute incubation with DAMGO, the drug was washed out and replaced with media containing antagonist to prevent subsequent activation of MOR and promote recycling. When latA or cytochalasin D were added to the washout media to destabilize actin, SpH-MOR whole cell fluorescence did not recover as rapidly or to the same degree as the control washout (Fig 3-1 G). Together, these results suggest that MOR recycling is partially dependent on actin, and this requirement is regulated by MOR PKC phosphorylation sites, S363 and T370.



#### Figure 1. MOR PKC sites, S363 and T370 regulate actin-dependent recycling of MOR

A. Example image of SpH-MOR imaged using TIRF-M at 10 Hz. Arrow denotes a MOR exocytic event. Scale bar is 5 µm. B. Lifetime of a MOR endocytic event with surface plot of maximum intensity fluorescence (low fluorescence in purple and high fluorescence in white). Scale bar is 2.5 µm. C. Maximum intensity trace of exocytic (black) and endocytic (red) events. Max intensity of exocytic events peaks and decays rapidly, while exocytic events persist for several seconds to a minute. D. Kymographs of MOR-WT, MOR-S363A, and MOR-T370A with DAMGO only and after latA. E. Mean percent change in number of exocytic events across multiple cells, with initial rates with DAMGO normalized to 100%. MOR-WT percent exocytic events decreases by half, while MOR-S363A is unaffected by latA treatment. MOR-T370A decreases slightly more than WT with latA. Bars are mean across multiple cells, error is SEM. P values comparing DAMGO and latA in WT, S363A, and T370A were calculated from raw number of recycling events across multiple cells and are paired t tests. P value comparing WT latA to T370A is an unpaired t test. n (WT) = 20 cells, n (S363A) = 25 cells, n (T370A) = 15 cells. F. Example montage of SpH-MOR cells imaged in multifield confocal imaging assay to measure total cell fluorescence. G. MOR surface levels do not increase as much as the control washout when latA or cytoD was added to the washout. Data points are average across 30 fields of cells. Error bars are s.e.m.

#### MOR S363 regulates MOR sorting to distinct endosomes

To test if MOR S363 and T370 regulate MOR recycling at the level of endosomal sorting, we used live cell confocal fluorescence imaging to resolve MOR trafficking to endosomal compartments in HEK 293 cells stably expressing FLAG-tagged MOR. Prior to agonist stimulation, fluorescent anti-FLAG-labeled MORs were localized primarily to the cell surface (Fig 3-2 A). After addition of DAMGO, MORs were redistributed to endosomes, and tubule domains were observed protruding from MOR endosomes (Fig. 3-2 A), domains that have been shown to be associated with GPCR and membrane protein recycling (Maxfield and McGraw, 2004; Puthenveedu et al., 2010; Vistein and Puthenveedu, 2013). Unlike the wild type receptor, endosomes containing tubular domains were not detected in MOR-S363A cells (Fig 3-2 B), while MOR T370A localized to morphologically similar endosomes with tubular domains as MOR-WT (Fig 3-4). To characterize the morphology of MOR S363A endosomes, we calculated the total number and size of endosomes across multiple cells at 5, 10, 15, and 20 minutes after DAMGO activation of MOR. Total number of endosomes per cell was similar between MOR-WT, MOR S363A, and MOR T370A until 20 minutes after DAMGO, when the number of MOR S363A endosomes was approximately twenty percent higher than MOR WT and MOR T370A (Fig 3-2 C). The average endosome diameter of MOR S363A endosomes was significantly smaller than MOR WT and MOR T370A endosomes at all time points after DAMGO addition, and MOR T370A endosome diameter was larger than MOR WT at 15 and 20 minutes post-DAMGO (Fig 3-2 D). These data suggest that PKC phosphorylation of MOR could regulate MOR sorting to morphologically distinct endosomal compartments.



#### Figure 3-2. MOR S363 regulates MOR sorting to distinct endosomes

**A**. Example image of FLAG-MOR-WT before DAMGO and 20 minutes after DAMGO. After DAMGO addition, MOR localizes to endosomes (arrows) with tubular protrusions (arrowheads). Scale bar is 5  $\mu$ m. **B**. Example image of FLAG-MOR-S363A before DAMGO and 20 minutes after DAMGO. After DAMGO addition, MOR-S363A localizes to endosomes (arrows) that appear smaller than MOR-WT endosomes and without detectable tubular protrusions. Scale bar is 5  $\mu$ m. **C**. Average number of endosomes per cell for MOR-WT, S363A, and T370A. Error bars are s.e.m. \*\*\*\* = p value < 0.0001. n (MOR-WT) = 25 cells; n (MOR-S363A) = 31 cells; (MOR-T370A) = 15 cells. **D**. Average endosome diameter in microns across multiple cells for MOR-WT, S363A, and T370A. Error bars are s.e.m. \*\*\*\* = p value < 0.0001. n (MOR-WT) = 273 endosomes 5 min, 229 endosomes 10 min, 216 endosomes 15 min, 193 endosomes 20 min; n (MOR-S363A) = 222 endosomes 5 min, 312 endosomes 10 min, 225 endosomes 15 min, 278 endosomes 20 min; (MOR-T370A) = 266 endosomes 5 min, 147 endosomes 10 min, 164 endosomes 15 min, 196 endosomes 20 min.

# PKC phosphorylation of MOR at S363 regulates MOR trafficking to Rab4 and Rab11 endosomes.

To ask if the MOR PKC site, S363, regulates MOR trafficking to distinct endosomes, we imaged FLAG-MOR with markers of early and recycling endosomes, Rab4 and Rab11, machinery that have been shown to regulate GPCR recycling, including that of MOR (Seachrist and Ferguson, 2003; Wang et al., 2008; Yudowski et al., 2009). 5 minutes after DAMGO addition, we observed FLAG-MOR in endosomal compartments that contained Rab4-GFP (Fig 3-3 A). Quantitation of MOR intracellular spots that overlapped with Rab4 showed that approximately 50 percent of MOR localized to Rab4 endosomes after DAMGO (Fig 3-3 C). We observed more MOR S363A endosomes that did not colocalize with Rab4 than MOR WT (Fig 3-3 B). Colocalization analysis showed that approximately 20% of MOR S363A spots colocalized with Rab4 after DAMGO (Fig 3-3 C). Colocalization of MOR S363A with Rab11 was slightly lower than that of MOR WT, but was only significantly different at 15 and 20 minutes after DAMGO (Fig 3-3 D-F). MOR T370A colocalization with Rab4 and Rab11 was not significantly different from MOR WT (data not shown). Together, these results suggest that phosphorylation of MOR at S363 regulates MOR trafficking to Rab4 and Rab11 endosomes.



## Figure 3. PKC phosphorylation of MOR at S363 regulates MOR trafficking to Rab4 and Rab11 endosomes.

**A**. Example images of MOR-WT and Rab4 GFP, scale bar is 5  $\mu$ m. **B**. Example images of MOR-S363A and Rab4 GFP, scale bar is 5  $\mu$ m **C**. Percent of MOR-WT and MOR-S363A spots that overlap with Rab4 GFP, analyzed using Imaris imaging software colocalize spots algorithm. Bars represent the average across multiple cells, error bars are s.e.m. n (MOR-WT) = 36 cells; n (MOR-S363A) = 22 cells. P values were calculated across the number of endosomes per cell in multiple cells and are unpaired t tests. **D**. Example images of MOR-WT and Rab11 RFP, scale bar is 5  $\mu$ m. **E**. Example images of MOR-S363A and Rab11 RFP, scale bar is 5  $\mu$ m. **F**. Percent of MOR-WT and MOR-S363A spots that overlap with Rab11 RFP, analyzed using Imaris imaging software colocalize spots algorithm. Bars represent the average across multiple cells, error bars are s.e.m. n (MOR-WT) = 15 cells; n (MOR-S363A) = 13 cells. P values were calculated across the number of endosomes per cell in multiple cells and are unpaired t tests.

# Phosphorylation of MOR at T370 switches MOR sorting between endosomal microdomains.

Because MOR recycling is dependent on actin, we next asked if PKC phosphorylation of MOR regulates MOR sorting into endosomal ASRT domains, that have been shown to mediate GPCR sequence-dependent recycling in the case of B2AR (Lauffer et al., 2010; Puthenveedu et al., 2010; Temkin et al., 2011; Vistein and Puthenveedu, 2013). Live cell imaging of FLAG-MOR with the cortical actin marker of endosomal ASRT domains, coronin-GFP, revealed MOR endosomal tubules that contain actin (Fig 3-4 A). Interestingly, unlike the B2AR, which is exclusively localized to ASRT domains, MOR localized to both actin-containing tubules and endosomal tubules that were not marked by coronin (Fig 3-4 A-B), consistent with our result that MOR recycling is only partially dependent on actin (Fig 3-1). In contrast, MOR T370A was localized exclusively to coronin tubules (Fig 3-4 C). Quantitation of the percent of total tubules per cell that contain coronin revealed that half of MOR WT localizes to coronin tubules, while MOR T370A localizes only to coronin tubules (Fig 3-4 D). Interestingly, this suggests that the drug-selective phosphorylation site, T370, regulates sorting of MOR between distinct endosomal microdomains.



### Figure 4. Phosphorylation of MOR at T370 switches MOR sorting between endosomal microdomains.

**A.** Example images of FLAG-MOR and coronin-GFP 20 minutes after DAMGO. MOR endosomal structures are visible (arrows) with tubule protrusions (arrowheads), both with coronin spots (left endosome) and without coronin spots (right endosome). Scale bar is 5  $\mu$ m. **B**. Inset of endosome examples from panel A, showing tubule with coronin (left) and without coronin (right). Scale bar is 2  $\mu$ m. **C**. Example images of MOR-WT (left panel) and MOR-T370A (right panel) endosomal microdomains. MOR localizes to tubules with (arrowhead) and without (arrow) coronin. Scale bar is 2  $\mu$ m. **D**. Quantitation of percent total tubules per cell that have (+coronin) or do not have coronin (-coronin). Bars are average across multiple cells, error bars are s.e.m. n (MOR-WT) = 25 cells ; n (MOR-T370A) = 18 cells. P value was calculated across raw tubule number per cell for multiple cells and is an unpaired t test.

# MOR T370A upregulates expression of genes downstream of endosomal cAMP production.

Recent advances in GPCR signaling assays and live cell imaging have allowed for direct visualization of activation of the Ga stimulatory protein on endosomes containing B2AR (Irannejad et al., 2013). Interestingly, activation of adenylyl cyclase at the cell membrane or endosomal membranes upregulates expression of distinct downstream gene targets (Tsvetanova and Zastrow, 2014), providing a functional readout of cyclic AMP (cAMP)-dependent endosomal signaling. This also suggests that membrane and endosomal G protein signaling cause distinct downstream consequences that could lead to different overall cell responses to signals. Further, PKA phosphorylation of the B2AR switches B2AR between endosomal ASRT and constitutive recycling microdomains, and B2AR localization to ASRT endosomal domains results in an increase in expression of the cAMP-dependent endosomal signaling gene target, PCK1 (Chapter 1). B2AR recruited active Gas exclusively from ASRT domains, not constitutive recycling tubules (Chapter 1), suggesting that endosomal ASRT domains may function as G protein signaling scaffold domains. Therefore, we asked if PKC-regulated sorting of MOR into ASRT domains also controls generation of gene products downstream of endosomal cAMP production.

Agonist activation of MOR stimulates coupling of MOR to the Ga inhibitory protein (Ga<sub>i</sub>), resulting in inhibition of adenylyl cyclase and a decrease in cAMP production (Hsia et al., 1984; Jordan and Devi, 1998; Sharma et al., 1975). Therefore, we hypothesized that if MOR activates Ga<sub>i</sub> from endosomes, expression of the endosomal cAMP-dependent gene, PCK1 should decrease, and PCK1 expression

should decrease more for MOR-T370A when the receptor is exclusively localized to ASRT domains. First, we tested the stability of reference gene targets, beta tubulin (TUBB) and hypoxanthine phosphoribosyltransferase 1 (HPRT1), in the presence and absence of DAMGO using quantitative real-time PCR. The threshold cycle (Ct) at which TUBB and HPRT1 were amplified did not significantly change with DAMGO addition for either MOR-WT or MOR-T370A (Fig 3-5 A-D), confirming that TUBB and HPRT1 are suitable reference genes for normalizing PCK1 expression across treatments. DAMGO addition did not significantly decrease PCK1 expression in MOR WT cells (Fig 3-5 E, G). However, activation of MOR T370A cells with DAMGO decreased PCK1 expression (Fig 3-5 F) by approximately fifty percent on average (Fig 3-5 G). These data suggest that PKC-regulated sorting of MOR into actin-dependent tubules could potentially serve to generate an endosomal G protein signaling bias.



Figure 5. Phosphorylation of MOR at T370 switches MOR sorting between endosomal microdomains.

A. Paired comparisons of raw threshold cycle number amplification (Ct) detected by SYBR green fluorescence increase over background threshold for TUBB mRNA. Pairs compare no treatment vs. DAMGO for both MOR-WT (black circles). TUBB Cts did not significantly change with DAMGO addition. Each pair represents one experimental replicate for all following panels (n = 4). B. Paired comparison with DAMGO for MOR-T370A. TUBB Cts did not significantly change with DAMGO addition. C. Paired comparisons of raw threshold cycle number of amplification (Ct) detected by SYBR green fluorescence increase over background threshold for HPRT1 mRNA. HPRT1 Cts did not significantly change with DAMGO addition. D. Paired comparison with , DAMGO for MOR-T370A. HPRT1 Cts did not significantly change with DAMGO addition. E. Paired comparisons of raw threshold cycle number of amplification (Ct) detected by SYBR green fluorescence increase over background threshold for PCK1 mRNA. PCK1 Cts did not significantly change with DAMGO addition for MOR-WT. F. Paired comparison with DAMGO for MOR-T370A. PCK1 Ct numbers increased with DAMGO addition, indicating that PCK1 was amplified at a later cycle with DAMGO, indicative of a DAMGO-induced decrease in PCK1 expression. P values for all paired comparisons were calculated across multiple experimental replicates and are unpaired t tests. G. Fold change in expression of PCK1 with DAMGO for MOR-WT and MOR-T370A. Expression was normalized to TUBB Cts for all samples. PCK1 expression decreased with DAMGO for MOR-T370A but not MOR-WT. Black bars represent mean across all experimental replicates, error is SEM across experimental replicates (n = 4 for all samples).

#### Discussion

We show that MOR endosomal sorting undergoes hierarchical regulation by PKC phosphorylation of MOR. The constitutively phosphorylated site, S363, regulates sorting of MOR to Rab4 and Rab11 endosomal compartments. The drug selective phosphorylation site, T370, switches MOR sorting between endosomal microdomains. Restriction of MOR to endosomal actin microdomains when T370 is not phosphorylated may serve to generate an endosomal signaling bias, suggesting that drug-selective phosphorylation of GPCRs could function to regulate receptor activity by changing endosomal sorting.

Hierarchical sorting of a GPCR has been demonstrated for the B2AR. In the case of B2AR, adrenergic signaling activates PKA, which phosphorylates the receptor at serines 345 and 346. Dephosphorylation of these sites on B2AR induces sorting of the receptor into both constitutive recycling tubules and endosomal ASRT domains. This change in sorting alters the kinetics of B2AR recycling and resensitization to adrenergic signaling, suggesting that adrenergic signaling homologously regulates B2AR activity at the level of endosomal sorting. Interestingly, MOR, unlike B2AR does not localize exclusively to endosomal ASRT domains that mediate GPCR sequence-dependent recycling. This difference could arise from the diverse recycling sequences on B2AR and MOR, as MOR's sequence does not conform to a type I PDZ ligand like the recycling sequence on B2AR and several other GPCRs (Cao et al., 1999; Gage, 2001; Hanyaloglu et al., 2008; Romero et al., 2011; Tanowitz and Zastrow, 2003a). Interestingly, MOR recycling was still partially dependent on actin, and the mechanism for how its sorting sequences couples it to the actin cytoskeleton remains unknown.

Perhaps diverse GPCR recycling sequences function to recruit distinct trafficking machinery to different receptors that localize to the same endocytic compartments, allowing for specific regulation of the many physiological systems mediated by GPCRs. Hierarchical sorting of GPCRs by signaling kinases could serve to provide an extra level of regulation by changing endosomal sorting of GPCRs in response to rapid physiological signals.

MOR recycling has been shown to be dependent on both Rab4 and Rab11 (Wang et al., 2008). PKC phosphorylation was shown to switch MOR recycling from a Rab4- to a Rab11-dependent pathway (Wang et al., 2008). This study suggested that this PKC-mediated switch required three sites on the C-terminal tail of MOR, S363, T370, and S375. Our results suggest that it is possible that S363 alone may be needed to switch MOR recycling between Rab4 and Rab11 pathways. However, we did not investigate the role of S375 in localization of MOR to Rab4 and Rab11 compartments or if our single alanine mutants depend on either Rab4 or Rab11 function for recycling. Interestingly, phosphorylation of T370 is enhanced when S375 is already phosphorylated (Mann et al., 2015). This hierarchical dependence on S375 phosphorylation could allow the cell to fine tune changes in T370-mediated MOR sorting by controlling the fraction of MORs that are phosphorylated at this site.

Rapid recycling of MOR has been shown to be dependent on both actin and microtubules in medium spiny neurons from the striatum (MSNs) (Roman-Vendrell et al., 2012). Interestingly, this same study showed that MOR recycling is PKA dependent in MSNs, when MORs are activated by morphine (Roman-Vendrell et al., 2012). Previous work from our laboratory has shown that MOR recycling is not PKA-dependent

in HEK 293 cells (unpublished data, Amanda Soohoo). This difference in PKC and PKAdependent regulation of MOR could reflect differential regulation of GPCR sorting in diverse cell types. The existence of distinct hierarchical sorting mechanisms in different cell types could allow for differential regulation of receptors whose trafficking shares many of the same core trafficking machinery.

Our data suggest that the drug selective phosphorylation site, T370, regulates MOR sorting into endosomal ASRT domains. Agonist-dependent regulation of GPCR endosomal sorting has not yet been demonstrated for other GPCRs, but further studies of the mechanisms of GPCR endosomal sorting could help us better understand how different drugs regulate the same GPCR to cause distinct effects. Interestingly, T370 can be phosphorylated by both GRK and PKC, and heterologous PKC phosphorylation of T370 downstream of the pain sensing neurotransmitter, substance P, has been demonstrated (Mann et al., 2015). The agonist selective and heterologous phosphorylation profile of this site could provide a way for the cell to differentially regulate MOR trafficking and activity to mediate diverse effects of opiates.

Our results suggest a potential role for PKC-regulated endosomal sorting of MOR in generating an endosomal signaling bias. While it remains unclear if Ga<sub>i</sub> is directly activated from endosomal membranes, there is growing evidence that both Ga<sub>s</sub> and Ga<sub>q</sub> are activated by GPCRs from endosomes (Calebiro et al., 2009, 2010; Ferrandon et al., 2009; Irannejad et al., 2013; Okazaki et al., 2008; West and Hanyaloglu, 2015). Surprisingly, we only observed a decrease in expression of the cAMP-dependent gene, PCK1, in MOR-T370A cells, but not by the wild type receptor. One explanation for this result is that the decrease in PCK1 expression induced when only half of MORs are in

endosomal ASRT domains is below the detection level of our gene expression measurements. Another possibility is that MOR does not couple to Gq<sub>i</sub> in non-ASRT domains, or MOR may not exist in an activated conformation in the entire endosome. The role of hierarchical endosomal sorting and the ASRT machinery in regulating GPCR endosomal G protein signaling requires further investigation. Further studies of the function of downstream targets of endosomal signaling and high resolution imaging of GPCR endosomal sorting will help expand our understanding of the role of membrane trafficking in regulating receptor activity.

#### **Experimental Procedures**

#### Constructs and Reagents

FLAG-MOR, FLAG-MOR-S363A, FLAG-MOR-T370A, cortactin, and sorting nexin-1 constructs have been described previously (Bowman et al., 2015a; Hanyaloglu et al., 2005; Puthenveedu et al., 2010; Vistein and Puthenveedu, 2013). Rab4-GFP and Rab11-RFP plasmid constructs were generously donated to us by Mark von Zastrow and described previously (Yudowski et al., 2009). HEK293 cells were purchased from ATCC and cultured in DMEM High Glucose (Fisher Scientific-HyClone) + 10% FBS (Gibco). Cells were transfected with Effectene (QIAGEN). Stable cell lines of FLAG-MOR, FLAG-MOR-S363A, FLAG-MOR-T370A were obtained with Geneticin (Invitrogen) selection. Cells were passed to 25 mm coverglass 1 day after transfection with actin or Rab markers, and imaged the next day (48 hours post transfection).

[D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) and naloxone (Sigma-Aldrich) were prepared as 10 mM stocks in water. Latrunculin A (latA) (Cayman Chemical) was obtained at a concentration of 100  $\mu$ g/mL in ethanol. Cytochalsin D was prepared at 10

mM stock (Sigma-Aldrich). DAMGO, naloxone, latA, and cytochalasin D (cytoD) were used at a final concentration of 10  $\mu$ M.

#### Microscope Image Acquisition and Analysis

Confocal images were acquired with an Andor Revolution XD spinning disk system on a Nikon Eclipse Ti inverted microscope equipped with a temperature-, humidity-, and CO2-controlled chamber and a 100x total internal reflection fluorescence (TIRF) objective (Nikon). Cells were imaged live at 37 °C in Leibovitz's L15 - phenol red (Gibco) with 1% or 5% FBS. Solid-state 488-nm, 561-nm, or 647-nm lasers served as light sources. Images were acquired with an iXon+ 897 EMCCD camera. HEK 293 cells stably expressing FLAG-MOR constructs were labeled with M1 anti-FLAG conjugated to Alexa-647 (Molecular Probes) at 37 °C, in DMEM high glucose + 10% FBS. Cells were imaged 5–20 min after the addition of DAMGO. All fluorescence measurements and quantitations were performed on images acquired directly from the camera without adjustments.

#### Quantitation of Individual Recycling Events

Cells stably expressing SpH-MOR, SpH-MOR S363A, or SpH-MOR T370A were plated on coverslips and imaged 24 hours later. Coverslips were stimulated with DAMGO and imaged 5 minutes later. The same coverslip was then incubated with latrunculinA and imaged 5 minutes later. Data show the average number of individual recycling events per minute with the DAMGO only (before latrunculinA) movie normalized to 100 percent. Experiments were repeated at least 3 separate times, and data show the mean across all cells from all experimental replicates. Paired t tests were calculated across the raw number of individual recycling events per minute, comparing DAMGO only (before latrunculin A) and after latrunculin A was added.

#### Quantitation of Tubule Number Per Cell

HEK 293 cells stably expressing FLAG-tagged MOR or MOR-T370A were transiently transfected with coronin-GFP and imaged 48 hours after transfection. Images were acquired 5-10 minutes after DAMGO addition. The total number of MOR positive tubules per cell that colocalized with coronin-GFP (ASRT marker) were manually counted double blind with scrambled file names. Experiments were performed at least three separate times for both MOR and MOR-T370A. Plotted data show results across all cells from each experimental replicate, and unpaired student's t tests were performed.

#### Multifield Recycling Assay

Cells were imaged in confocal with a 20X objective, and 10 XY fields were collected at 1 image per minute. A 5 min baseline was captured before DAMGO was added to cells. Cells were imaged with DAMGO for 10 minutes, and DAMGO media was washed out twice and replaced with media containing the MOR antagonist, naloxone to prevent subsequent activation of MOR and prevent recycling. Cells were treated with either naloxone only, or naloxone + latA or cytoD. Whole field fluorescence was measured for all XY fields. Experiments were performed 2 separate times with 10 XY fields collected for each coverslip, and data show the average whole field fluorescence intensity across XY fields from all experimental replicates. *Quantitation of Endosome Size, Number, and Colocalization in Imaris Imaging Software* 

Cells stably expressing FLAG-MOR WT, FLAG-MOR S363A, or FLAG-MOR T370A were transiently transfected with Rab4-GFP or Rab11-RFP and imaged 48 hours later. Cells were stimulated with DAMGO and images were acquired at 5 minutes, 10

minutes, 15 minutes, and 20 minutes after DAMGO addition. Experiments were performed 2 separate times, and data show the mean across all cells from all experimental replicates. Endosome size, number, and colocalization of MOR endosomes with Rab4 and Rab11 markers were quantitated using the Imaris imaging software spot detection algorithm and colocalize spots Imaris Xtension algorithm. *RNA isolation, Reverse Transcription, and Quantitative, Real-time-PCR.* 

RNA was harvested from HEK 293 cells 2 hours after DAMGO addition, using the RNeasy Plus Mini Kit (Qiagen). Cells were co-treated with latA and iso for 2 hours. Cells were pre-treated with dynasore for 20 minutes, and then treated with iso for 2 hours before RNA isolation. RNA was treated with DNase I, amplification grade (Life Technologies), and reverse transcription was performed with the Superscript First Strand System for RT-PCR (Life Technologies), RNA was amplified using random hexamer primers (Life Technologies). cDNA was then treated with RNase H (Life Technologies) prior to qRT-PCR reactions. Experiments were repeated 2 separate times with 2 replicates of each condition within each experiment. Plotted data show results across all replicate wells across all experimental replicates, and unpaired student's t tests were performed. Primers for gene targets were chosen to amplify targets 50-100 bp and spanning an exon-exon junction. The following primers were used to amplify gene targets: TUBB: Forward: 5'-GTGGTACGGAAGGAGGTCGATG-3'; Reverse: 5'-AAGGTGACTGCCATCTTGAGG-3'; HPRT1: Forward: 5'-GAACCTCTCGGCTTTCCCG-3', Reverse: 5'-CACTAATCACGACGCCAGGG-3'; PCK1: Forward: 5'-CTGCCCAAGATCTTCCATGT-3', Reverse: 5'-CAGCACCCTGGAGTTCTCTC-3'. TUBB and HPRT1 were chosen as reference

("house-keeping") genes for normalization because they did not change significantly

with iso addition. The SYBR Green Select Master Mix (Life Technologies) was used for

qRT-PCR amplification and detection on a BioRad Cfx RT PCR machine.

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### Chapter 4: Cell-autonomous regulation of mu-opioid

### receptor recycling by substance P

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#### Abstract

How neurons coordinate and reprogram multiple neurotransmitter signals is an area of broad interest. Here we show that substance P (SP), a neuropeptide associated with inflammatory pain, reprograms opioid receptor recycling and signaling. SP, through activation of the neurokinin-1 receptor, increases the post-endocytic recycling of MOR in trigeminal ganglia (TG) neurons in an agonist-selective manner. SP-mediated Protein kinase C (PKC) activation was both required and sufficient for increasing recycling of exogenous and endogenous MOR in TG neurons. The target of this cross-regulation was MOR itself, as mutation of either of two PKC phosphorylation sites on MOR abolished the SP-induced increase in recycling and resensitization. Further, SP enhanced the resensitization of fentanyl-induced, but not morphine-induced, antinociception in mice. Together, our results define a physiological pathway that cross-regulates opioid receptor recycling via direct modification of MOR, and suggest a novel mode of homeostatic interaction between the pain and analgesic systems.

#### Introduction

Most neurotransmitter signals are transduced by G protein-coupled receptors (GPCR), the largest family of signaling receptors (Pierce et al., 2002; Rosenbaum et al., 2009; Premont and Gainetdinov, 2007; Shepherd and Huganir, 2007; von Zastrow and Williams, 2012). The strength of a neuronal response directly depends on surface receptor numbers. Therefore, regulation of this number via membrane trafficking is critical for modulating neuronal responsiveness to a given signal (Anggono and Huganir, 2012; Gainetdinov et al., 2004; Marchese et al., 2008; Yudowski et al., 2009). It is accepted that membrane trafficking can control the number of surface receptors and therefore signaling, and many mechanisms have been identified. Emerging evidence suggests that signaling can also control membrane trafficking, but the mechanisms that underlie such crosstalk are still largely unresolved (Jean-Alphonse and Hanyaloglu, 2011).

Post-endocytic receptor sorting, a trafficking step critical for receptor physiology (Sorkin and von Zastrow, 2009; Anggono and Huganir, 2012; Marchese et al., 2008; Scita and Di Fiore, 2010; Williams et al., 2012), provides a potential point for such crosstalk. Activated surface receptors are rapidly internalized by clathrin-mediated endocytosis and transported to the endosome, causing receptor removal from the cell surface, which is associated with loss of cellular sensitivity (Alvarez et al., 2002; Claing et al., 2002; Hanyaloglu and Zastrow, 2007; Keith et al., 1996; Martini and Whistler, 2007). Cellular sensitivity to further extracellular signals is then determined by post-endocytic receptor sorting between the degradative and recycling pathways, as small changes in recycling rates can cause relatively large changes in surface receptor

numbers over physiological timescales (Sorkin and von Zastrow, 2009; Arttamangkul et al., 2012; Jean-Alphonse and Hanyaloglu, 2011; von Zastrow and Williams, 2012). How receptor recycling is controlled by heterologous signaling pathways in a physiological context is a fundamental question that is still not very well understood (Marchese et al., 2008; Williams et al., 2012).

Here, we focused on two signaling pathways that functionally interact - pain and analgesia - as physiologically relevant examples for potential signaling crosstalk. Pain in nociceptive neurons is associated with activation of the neurokinin-1 receptor (NK1R) by substance P (Perl, 2007; de Felipe, 1998), while analgesia is primarily mediated by opioids via the mu opioid receptor (MOR) (Chen and Marvizón, 2009; Kieffer, 1995; Lao et al., 2008). Here we show that NK1R activation by substance P increases MOR postendocytic recycling in sensory neurons, via a novel cross-regulatory mechanism based on direct modification of MOR. NK1R signaling also increases the resensitization of MOR-mediated antinociception in mice. Our results provide a physiologically relevant example for crosstalk between signaling pathways at the level of receptor trafficking.

#### Results

#### Substance P signaling through NK1R increases post-endocytic recycling of MOR

To test if NK1R signaling cross-regulates MOR recycling, we chose trigeminal ganglia (TG) neurons as model cells. TG neurons are highly relevant for neuralgia, a common and severe pain disorder, and they endogenously express MOR and NK1R (Aicher et al., 2000). To measure MOR recycling, we used an assay to quantitate

recycled FLAG-tagged MORs (Fig 4-1A). These tagged receptors were fully competent for signaling and trafficking, as reported previously (Arttamangkul et al., 2008; Just et al., 2013; Keith et al., 1996; Soohoo and Puthenveedu, 2013). TG neurons expressing FLAG-MOR were labeled with fluorescent Alexa 488-conjugated anti-FLAG antibodies to detect the existing pool of MOR on the cell surface (Fig 4-1B, surf ctrl). MOR activation by the specific agonist [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO, noted as DG) induced robust MOR internalization, detected by the appearance of intracellular MOR fluorescence (Fig 4-1B, intern). DG was then washed out to allow MOR recycling. The cells were then labeled by Alexa 568-conjugated secondary antibodies, which only label surface anti-FLAG-labeled MOR. MOR recycling was quantitated as the ratio of the secondary (surface) to primary (total) antibody fluorescence values. This ratiometric assay allowed us to differentiate recycling from the insertion of newly synthesized MOR. Activation of endogenous NK1Rs by substance P (SP) during the agonist washout increased the ratio of surface to total fluorescence, indicating increased MOR recycling (Fig 4-1B and C, rec vs. rec + SP) (Hunt and Mantyh, 2001; Nichols et al., 2014; de Felipe et al., 1998). We measured the pixelbased colocalization of the surface to total MOR by calculating the Pearson's correlation between the two fluorophores. Before DG, a strong correlation was observed as seen in the cytofluorogram (e.g. cell in Fig 4-1D-E. After DG, colocalization decreased and two separate populations emerged, consistent with MOR endocytosis and decreased labeling with the secondary antibody on the surface (Fig 4-1D and E). Colocalization increased when SP was added to the washout (Fig 4-1E), suggesting an increase in surface MOR. We next asked if SP was capable of regulating MOR recycling when



Fig 4-1. Substance P increases post-endocytic recycling of MOR. A. Schematic of the quantitative ratiometric recycling assay in TG neurons. B. Example images of FLAG-MOR in primary TG neurons: surface control (surf ctrl), internalization control (intern), DG washout (rec), and washout with SP (rec + SP). Primary anti-FLAG labeling, showing the total pool of receptors initially on the surface (total 488) is green in the overlay. Secondary antibody labeling showing the fraction of the initial pool on the surface at the end of the treatment (surf 568) is red in the overlay. A ratio of the 568 to 488 fluorescence gives the fraction of the initial pool present on the surface at the end. Scale bar is 10 µm C. Percent recycling calculated from the ratios of surface (568) to total (488) in each condition (mean ± s.e.m; n = 77 surf ctrl, 78 intern, 69 rec, and 68 rec + SP). D. Cytofluorograms showing pixel-level fluorescence correlation between total and surface-only pools. The surf ctrl shows strong correlation between the two channels; the intensity values trend to a single diagonal line. DG (intern) caused separation of the points into two populations, consistent with a decrease in colocalization because the anti-FLAG on internalized MOR is not accessible to the secondary antibodies. DG washout increased the correlation, which was further enhanced by SP. Pearson's correlation coefficients are shown for each example. E. Tukey box plots showing the 1st and 3rd guartiles of the distribution of Pearson's coefficients across multiple cells (n as above). Middle bar shows the median, outside bars show 10th and 90th percentiles, and "+" shows the mean. Scale bars are 5µm. F. Percent recycling increased during washout, following activation of TGs with fentanyl. DG (n = 37 surf ctrl, 33 intern, 29 rec, and 38 rec + SP). G. Tukey box plots showing Pearson's coefficients from cells in F. H Percent recycling increased during washout, following activation of TGs with morphine. (n = 37 surf ctrl, 34 intern, 37 rec, and 30 rec + SP). Tukey box plots showing Pearson's coefficients from cells in H.

MORs were stimulated with two clinically relevant opioids, fentanyl and morphine. SP increased MOR recycling after endocytosis induced by fentanyl (Fig 4-1 F-G, but not morphine (Fig 4-1 H-I).

To directly visualize and quantify MOR recycling at the level of individual recycling events (Yudowski et al., 2006), we imaged MOR N-terminally tagged with a pH-sensitive green fluorescent protein (SpH-MOR) (Miesenböck et al., 1998). When expressed in HEK293 cells, MOR fluorescence was quenched in acidic endosomal compartments and dequenched upon recycling (Yudowski et al., 2009). Rapid imaging (10 Hz) using total internal reflection fluorescence microscopy (TIR-FM) (Puthenveedu et al., 2010; Yu et al., 2010) after MOR endocytosis revealed individual exocytic events as transient bursts of fluorescence at the cell surface (Fig 4-2A). The fluorescence burst showed a localized peak of maximum intensity that diffuses across a larger area as vesicles fuse and receptors diffuse across the cell surface (Fig 4-2B, heat map of intensity shown below, Fig 4-2C), consistent with our previous data that these are individual recycling events (Puthenveedu et al., 2010; Yudowski et al., 2009).

We calculated the percentage change in the number of recycling events after SP by normalizing to the initial rate before SP (Fig 4-2D). In cells expressing HA-NK1R, the percentage of MOR recycling events per minute increased after SP (Fig 4-2E, F), consistent with the increase we saw with endogenous NK1R (Fig 4-1C, E). In adjacent cells not expressing HA-NK1R, SP did not increase SpH-MOR recycling (Fig 4-2F). As HEK293 cells do not express noticeable levels of endogenous NK1R, this indicates that NK1R signaling is sufficient to increase MOR recycling. MOR recycling was not reduced by cycloheximide treatment, confirming that these were post-endocytic recycling events,



#### Fig 4-2. Substance P signaling through NK1-R regulates individual MOR recycling events. A.

Cells expressing SpH-MOR imaged with TIR-FM 5 min after DG addition. Frames are 100ms apart. An individual exocytic event is indicated by yellow arrow. Scale bar is 5µm. **B**. Lifetime of an SpH-MOR exocytic event. Insertion events begin as a localized, intense burst of fluorescence that diffuses within a second. Heat map of intensity is shown below as a surface plot. **C**. Maximum intensity traces of an SpH-MOR vesicle exocytic event (top, arrows), showing characteristic rapid spikes, and an endocytic cluster (bottom), which persists for much longer with a characteristic exponential decrease at the end. **D**. Experimental workflow to quantify acute regulation of recycling. **E**. Kymographs of SpH-MOR fusion events from the same region in the same cell, expressing both SpH-MOR and an HA-tagged NK1-R, following sequential DG and SP addition. Arrowheads show exocytic events which increase after SP. Scale bar is 2 seconds **F**. Number of SpH-MOR exocytic events per min after SP addition normalized to before (i.e., with just DG) in cells expressing NK1-R and in adjacent cells not expressing NK1-R. Error bars are s.e.m. (n = 20). See also Fig S1, Movie S1, Movie S2, S3.

and not insertion of newly synthesized protein (Fig S4-1A). Additionally, very few MOR recycling events were seen without DG stimulation, and SP did not change this (Fig S4-1B). Together, our results show that SP signaling increases MOR recycling through activation of the NK1R.

# Protein Kinase C signaling is required and sufficient for SP-induced increase in MOR recycling and resensitization.

We next addressed the intracellular NK1R signaling cascade that mediated the regulation of MOR recycling. NK1R couples to Gq/11, which activates Protein Kinase C (PKC) (Macdonald et al., 1996). The PKC inhibitor chelerythrine (chel) abolished the SP-induced increase in MOR recycling in NK1R-expressing cells (Fig 4-3A), indicating that PKC was required for SP- and NK1R-mediated regulation of MOR recycling. Additionally, PKC activation by Phorbol 12-myristate 13-acetate (PMA) increased SpH-MOR recycling in the absence of NK1R and SP (Fig 4-3B), indicating that PKC was sufficient for increasing MOR recycling. Addition of chel or PMA alone had no effect on SpH-MOR exocytic events (Fig S4-2A-B).

To investigate the functional consequences of PKC-mediated regulation of MOR recycling, we first measured DG-mediated inhibition of cAMP levels as a readout of the number of functional surface MOR (Talbot et al., 2005). HEK293 cells expressing MOR were stimulated with DG for 15 min to induce MOR endocytosis and cellular desensitization. DG was washed out to allow MOR recycling, and cAMP inhibition in response to a rechallenge with DG measured as an index of cellular resensitization. Addition of chel during the washout decreased cAMP inhibition after the rechallenge



**Fig 4-3.** PKC activation is required and sufficient for SP-induced increase in MOR recycling. **A.** Percent change in number of MOR recycling events, comparing SP to SP + chel. Chel blocked the SP-mediated increase. DG and either SP or SP + chel were added sequentially, and a paired comparison was made in the same cell (n = 12). **B**. PMA was sufficient to increase MOR recycling (n = 47 cells). **C**. Assay to detect resensitization of cells to MOR signaling using luminescence-based real-time detection of cAMP levels. Addition of DG reduces cAMP levels (left). Following DG washout (+ chelerythrine or PMA) to allow recycling, cells were rechallenged with DG. Chel (green) reduced recycling of functional MOR, while PMA (blue) enhanced it compared to control cells (red). The initial DG challenge was identical across all experiments. Error bars are s.e.m. across 9 experiments. See also Fig S2.



Fig 4-4. PKC activation is required and sufficient for SP-induced increase in MOR recycling in TG neurons. A. Ratiometric recycling assay in TG neurons comparing MOR recycling in the presence of SP alone vs. SP with chel. Scale bar is 10  $\mu$ m. B. Quantitation across multiple cells, as in Fig 1C (mean ± s.e.m; n = 77 surf ctrl, 78 intern, 72 rec + SP, 47 rec + SP + chel). C. Tukey box plots of Pearson's coefficients from cells in B. D-E. Ratiometric recycling assay in TG neurons testing the effect of PKC activation by PMA in the absence of SP (n = 77 surf ctrl, 78 intern, 69 rec, 47 rec + PMA). F. Tukey box plots of Pearson's coefficients from cells in E. (green line) compared to the control (red line). In contrast, PMA increased cAMP inhibition in response to the DG rechallenge (blue line) (Fig 4-3C). Chel and PMA alone, with no prior DG stimulation, had no effect on DG induced inhibition of cAMP production (Fig S4-2C).

We next tested if PKC inhibition abolishes the SP-induced increase in MOR recycling in TG neurons, using the ratiometric recycling assay (Fig 4-1). Addition of chel during the washout abolished the SP-mediated increase in MOR recycling (Fig 4-4A, B). Pixel-based colocalization was lower when PKC was inhibited in the washout, even in the presence of SP (Fig 4-4C). Adding PMA, without SP, during the washout increased MOR recycling (Fig 4-4D-F). Together, this suggests that PKC is both required and sufficient for regulation of MOR recycling and cellular sensitivity to opioid signaling.

### Substance P and PKC-mediated regulation of MOR recycling requires MOR phosphorylation at Ser 363 and Thr 370

Considering that PKC was required and sufficient for heterologous regulation of MOR recycling through SP, we sought to identify the target of PKC. The MOR itself presented an interesting candidate. PKC can phosphorylate three sites on the C-terminal tail of MOR - serine 363, threonine 370, and serine 375 (Fig 4-5A) (Doll et al., 2011; Feng et al., 2011). To test whether MOR phosphorylation was required, we mutated each of these sites to alanine to block phosphorylation (Feng et al., 2011) and quantified SP-mediated regulation of MOR recycling. SP did not increase the percentage of recycling events per unit time when either S363 or T370 was mutated (Fig 4-5B, C). In contrast, the recycling of S375A increased to a level comparable to



**Fig 4-5. PKC requires S363 and T370 to regulate MOR recycling. A.** Schematic of PKC phosphorylation sites on C-terminal tail of MOR (S363, T370, and S375). **B**. Kymographs of SpH-MOR single exocytic events for MOR S363A, T370A, and S375A after SP. **C**. Quantitation of percent recycling across cells (n = 20, 14, 18, and 22) in HA-NK1R expressing cells with MOR mutants S363A and T370A, and S375, compared to wildtype. Dashed line shows number of events in same cells prior to SP normalized to 100%. **D**. Percent recycling with PMA-treated cells expressing MOR mutants S363A, T370A, and S375A (n = 52, 29, 18, and 17). **E**. Ratiometric recycling assay in TG neurons for S363A with and without SP. Scale bar is 10 µm. **F**. Ratio quantitation across multiple cells (mean  $\pm$  s.e.m; n = 35 surf ctrl, 38 intern, 33 rec, 41 rec + SP) between the washout without and with SP for S363A. **G**. Tukey box plots of Pearson's coefficients from S363A cells in F show no increase in correlation with SP. **H**. Ratiometric recycling assay in TG neurons for T370A with and without SP. **I**. Ratio quantitation (n = 47, 27, 29, and 27) without and with SP for T370A. **J**. Tukey box plots of Pearson's coefficients from T370A cells in I show no increase in correlation with SP. **m**.

wild type in response to SP (Fig 4-5B, C). This indicates that S363 and T370 are required for SP-mediated regulation, but S375 is not (Fig 4-5B, C). Additionally, PMA increased SpH-MOR exocytic events for S375A, but not S363A or T370A, comparable to wild type MOR (Fig 4-5D). In TG neurons, SP failed to increase S363A or T370A recycling (Fig 4-5E-G for S363A, and Fig 4-5H-J for T370A), indicating that both S363 and T370 are required for PKC to regulate MOR recycling.

#### PKC enhances recycling and resensitization of endogenous MORs in TG neurons.

We next asked if endogenous MOR trafficking was regulated by PKC. To test this, we utilized a rabbit monoclonal anti-MOR antibody (UMB-3), to detect the subcellular localization of endogenous MORs (Lupp et al., 2011). UMB-3 staining showed strong staining at the periphery of TG neurons (Fig 4-6A), further indicated by the surface plot of intensity (Fig 4-6A insets). To quantitate intracellular vs. membrane MOR levels, UMB-3 fluorescence was measured across concentric circles increasing in size from the center to the periphery of the cell (Fig 4-6B). At steady state, the majority of UMB-3 maximum fluorescence intensity was detected in larger circles, consistent with more MOR localized to the surface (Fig 4-6B). After DG addition, UMB-3 staining was visible in punctate structures, and fluorescence intensity was uniform across the cell (Fig 4-6A-B), suggesting redistribution of MOR to endosomes. DG washout restored UMB-3 staining at the periphery of TG neurons (Fig 4-6A-B), consistent with MOR recycling. PKC inhibition during the washout inhibited MOR recycling, as evidenced by retention of UMB-3 fluorescence in punctate structures and uniform fluorescence in smaller circles (Fig 4-6A-B). Conversely, PKC activation during the washout caused


Fig 4-6. PKC increases recycling of endogenous MOR and opioid resensitization in TG neurons. A. Example images of anti-MOR (UMB-3) in TG neurons. UMB-3 staining was primarily localized to the periphery in untreated cells (orange arrowheads). DG addition for 20 m induced a redistribution of UMB-3 staining to intracellular punctate structures (blue arrows). DG washout (20 m) induced greater UMB-3 staining at the periphery, similar to the untreated control. Addition of chel during the washout shifted UMB-3 staining to punctate structures, while PMA addition enhanced staining at the cell periphery. Insets show surface plots as heat maps. B. Schematic of radial profile method used to analyze fluorescence intensity of UMB-3 staining from the center to the periphery of cells. Intensity traces from multiple cells (mean ± s.e.m; n>8 in each condition) show increased UMB-3 fluorescence in circles of larger radii, consistent with increased MOR on the surface. Higher fluorescence in larger radii denotes surface, while uniform fluorescence denotes internal pools. C. Graph of average mean intensity of derm-A594 signal labeling endogenous MOR in TG neurons across 3 trials (mean ± s.e.m.) Derm-A594 fluorescence was significantly greater in non-treated TGs (no treatment) than HEK cells (neg ctrl), indicating specific binding. DG induced internalization, as shown by lower fluorescence (black bars). Washout increased fluorescence (red). Chel blocked this increase in fluorescence (green) and PMA enhanced it (blue). D. Graphs of mean fluorescence values of DiBAC4(5) in TG neurons, at 15 m after DG addition and DG rechallenge after the washout alone (left), with chel (center) and with PMA (right). Corresponding p values (mean  $\pm$  s.e.m; n>5 in each condition) are shown for each.

strong UMB-3 staining at the cell periphery (Fig 4-6A-B), suggesting that PKC increases endogenous MOR recycling.

To further test PKC's regulation of endogenous MOR recycling in TG neurons, we used a fluorescent ligand, Alexa 594-conjugated dermorphin (derm594), previously described to bind MORs (Arttamangkul et al., 2000). To induce recycling, we treated TG neurons with DG, followed by a washout as in the resensitization experiment in Fig 4-3C. At the end, the cells were labeled with ice-cold derm594 to detect surface MOR. When compared to the control, DG significantly decreased derm594 fluorescence, consistent with MOR endocytosis. After washout, derm594 fluorescence was higher than the DG control, as expected after MOR recycling. PKC inhibition decreased derm594 fluorescence, and PKC activation increased it, suggesting that PKC increases recycling of endogenous MOR (Fig 4-6C). HEK 293 cells not expressing MOR did not show fluorescence, confirming specificity of derm594 binding (Fig 4-6C). Together, these results suggest that PKC is required and sufficient to regulate recycling of endogenous MORs.

#### SP and PKC regulate the opioid resensitization in neurons and opioid analgesia in mice.

We next asked if PKC regulated the resensitization of opioid activity in physiologically relevant sensory neurons. TG neurons were incubated with the sulfonyl voltage-sensitive anionic dye DiBAC<sub>4</sub>(5) (George et al., 1998), which increases fluorescence on depolarization and decreases on hyperpolarization. DG decreased the fluorescence of KCI-activated TG neurons, consistent with opioid-induced hyperpolarization (Fig 4-6D). To measure MOR recycling and resensitization, we used

the agonist-washout paradigm above (Fig 4-6A-C). After the initial DG challenge, DG was washed out for 20 min to allow recycling and resensitization. A rechallenge with DG decreased the KCI-induced voltage change similarly to the initial challenge, indicating that neurons were resensitized to opioid signaling (Fig 4-6D, left graph). However, when PKC was inhibited during the DG washout, the DG rechallenge did not decrease fluorescence, consistent with fewer receptors recycling back to the surface (Fig 4-6D, middle graph). Further, PKC activation during the DG washout enhanced the effect of the DG rechallenge (Fig 4-6D, right graph). This suggests that PKC regulates opioid resensitization in sensory neurons, consistent with our model that SP-mediated PKC activation positively regulates MOR recycling and resensitization.

To test if SP regulated the resensitization of MOR-mediated analgesia in mice, we measured the development of acute tolerance to the antinociceptive effects of fentanyl, a short-acting MOR agonist, using a warm water tail-withdrawal assay (Melief et al., 2010; Pradhan et al., 2010) (Fig S4-3A). After baseline measurements, animals were injected with fentanyl, and tail-withdrawal latencies were measured every 30 min. A significant but sub-maximal increase in tail-withdrawal latencies, persisting for approximately 120 min, was observed with fentanyl (Fig 4-7A). Either saline (vehicle control) or SP was injected intrathecally 120 min after the first fentanyl challenge. In control mice, a fentanyl rechallenge, given 30 min later, attenuated (~40% of initial) the antinociceptive response, indicating acute tolerance to fentanyl (Fig 4-7A). In contrast, SP-injected mice showed an antinociceptive response to the rechallenge that was comparable to the initial response (Fig 4-7A, Fig S4-3B). Calculation of the areas under the curve showed that saline-injected mice showed a significantly reduced response to

the fentanyl rechallenge compared to the initial response, while SP-injected mice showed comparable responses to both fentanyl injections (Fig 4-7B). Because morphine-activated MORs were not subject to SP-regulated recycling, we next tested if SP could sensitize morphine-induced analgesia in mice. Consistent with our cellular data, a morphine rechallenge following SP injection did not increase tail withdrawal latency in contrast to fentanyl (Fig 4-7C-D, Fig S4-3C). Taken together, our results indicate that SP signaling through PKC inhibits acute tolerance to fentanyl, but not morphine, by increasing MOR recycling in peripheral neurons.





#### Discussion

We show that SP signaling, through endogenous NK1R, enhances MOR recycling following DG- and fentanyl-, but not morphine-induced endocytosis (Fig 4-1, 4-2). PKC activity downstream of NK1R is required and sufficient for this crosstalk (Fig 4-3, 4-4). We identify two PKC sites on the C-terminal tail of MOR as the targets for this NK1R-mediated regulation (Fig 4-5), and show a functional effect of PKC regulation in recycling of exogenous and endogenous MOR in sensory neurons (Fig 4-6). Further, we show that SP increases opioid antinociception in mice by attenuating acute tolerance to fentanyl, but not morphine.

Studies over the last decades have suggested a complex and paradoxical interaction between the neurokinin and opioid pathways. SP, a pain-associated neurotransmitter, can induce antinociceptive effects (Mohrland and Gebhart, 1979). Further, NK1R antagonists can modify opioid reward, withdrawal, and reinforcement, and NK1R is required for morphine reward but not morphine analgesia (Gadd et al., 2003; Murtra et al., 2000). Our data, that SP regulates MOR recycling and acute tolerance to fentanyl but not morphine (Fig 4-7), are consistent with this, but suggest a complex agonist-selective cross-talk between these pathways. At a cellular level, co-activation of NK1R and MOR in CNS neurons has been reported to inhibit MOR endocytosis, partly because NK1R non-specifically sequesters beta-arrestin, the common adapter required for GPCR endocytosis (Pierce et al., 2002; Yu et al., 2009), and partly because the receptors might heterodimerize (Pfeiffer et al., 2003). We directly measure individual recycling events which allows us to test acute regulation of MOR recycling induced by NK1R signaling in the same cell (Fig 4-2), free of the

potential confounding effect of NK1R on MOR endocytosis. Further, in our ratiometric assay, NK1Rs are activated after MOR is endocytosed, and the presence of a MOR antagonist prevents subsequent endocytosis. Therefore, we believe endocytosis has a negligible effect on the crosstalk we observe here. Further, blocking new MOR synthesis (Fig S4-1) had no effect on the surface delivery of MOR in our assays, and inhibition or activation of PKC without DG did not cause any change in MOR surface levels or induce surface insertion (Fig S4-2). Therefore, the increase in surface MOR we observe is primarily a result of increased recycling (Fig 4-1, 4-2).

The precise role of PKC in modulating opioid physiology and MOR trafficking is unresolved (Raehal et al., 2011; Williams et al., 2012), but it provides a potential control point for physiological regulation of opioid signaling. PKC has been implicated in controlling opiate resensitization, tolerance, and dependence, and PKC activation during prolonged MOR agonist exposure increases desensitization, possibly by endocytosis (Dang, 2004; Inoue and Ueda, 2000; Bailey et al., 2004; Kramer and Simon, 1999). As MOR itself can activate PKC, such homologous PKC activation during chronic MOR activation might regulate desensitization and endocytosis, while injury and inflammatory pain might alter the balance of MOR trafficking and resensitization through heterologous SP regulation, consistent with data that peripheral MORs are less active prior to injury or pain (Berg et al., 2007; Chen and Marvizón, 2009).

Such distinct cell-type or environment-dependent consequences could be brought about by differential MOR phosphorylation. Because we add SP after the major fraction of MOR is already internalized, we believe that the endosomal pool of MOR can be phosphorylated by PKC. Of the two MOR PKC targets required for SP-mediated

increase in MOR recycling, S363 is constitutively phosphorylated, while T370 phosphorylation is regulated. Interestingly, T370 is phosphorylated by DG, but not morphine (Doll et al., 2011). However, it is robustly phosphorylated by heterologous SP and PKC activity (Illing et al., 2014; Mann et al., 2014), so it is unlikely to be the primary explanation for the differences we see between DG and morphine. S375 might primarily be phosphorylated by GPCR Kinases (GRK) rather than PKC (Doll et al., 2012), consistent with our result that S375 is not required for NK1R's regulation of MOR recycling via PKC. Additionally, T370 might also be phosphorylated by GRK 2/3 following MOR activation with a hierarchical dependence on S375 phosphorylation (Just et al., 2013), and inhibition of GRK2 alleviates opiate tolerance (Dang et al., 2011). Further, substance P induces PKC-dependent phosphorylation of MOR at T370 without dependence on S375 phosphorylation (Illing et al, 2014). It is possible that T370 is differentially phosphorylated by PKCs and GRKs by homologous vs. heterologous regulation to control physiological consequences in different cell types. Homologous GRK-mediated phosphorylation of MOR following addition of opioid agonists might promote opioid tolerance in the CNS, while PKC phosphorylation at T370 following NK1R activation induces resensitization of MORs in the context of inflammatory pain in the PNS.

T370 and S363 are adjacent to a bi-leucine sequence that is required and sufficient for MOR recycling (Tanowitz and Zastrow, 2003). This raises the possibility that combinatorial MOR phosphorylation by homologous and heterologous signaling pathways might rapidly reprogram receptor recycling and cellular resensitization by changing the biochemical interactions of MOR. Reprogramming in response to

homologous regulation has been suggested for B2AR recycling, which switches between a sequence-dependent and bulk recycling pathway based on PKA phosphorylation (Puthenveedu et al., 2010; Vistein and Puthenveedu, 2013). MOR recycling in striatal neurons has been reported to be inhibited by forskolin, though PKA was not directly tested (Roman-Vendrell et al., 2012). Striatal neurons do not coexpress NK1R and MOR, and it is possible that different neuronal subtypes exhibit distinct mechanisms of regulation, depending on the expression profiles of signaling receptors and kinases. Rapid reprogramming by receptor phosphorylation could therefore be a general mechanism to switch receptors between different recycling pathways depending on the physiological circumstance. For MOR, such reprogramming, causing sensitization of nociceptors to opioid signaling, could in part explain the paradoxical analgesic effects of capsaicin and substance P.(Komatsu et al., 2009; Mohrland and Gebhart, 1979). We show that peripheral administration of substance P is capable of increasing MOR-mediated analgesia in mice. This is consistent with data that peripheral endogenous opioids are released following tissue damage and painful stimuli, and that this is accompanied by an increase in opioid receptors to nerve terminals (Stein and Lang, 2009). As the opioid system serves as a physiological check for the maladaptive consequences of pain, our results provide a proof of principle for how signaling crosstalk between these systems at the level of receptor trafficking could represent a general homeostatic mechanism of signaling crosstalk.

#### **Experimental Procedures**

#### Plasmid DNA and Constructs

FLAG-MOR and SpH-MOR have been described previously (Keith et al., 1996; Soohoo and Puthenveedu, 2013). HA-tagged rat NK1R was provided by Dr. Mark Von Zastrow. Point mutants were generated using site directed mutagenesis with QuikChange (Agilent). All constructs were confirmed by DNA sequencing.

#### Cell Cultures and Transfections

TG neurons were obtained as previously described (Malin, et al, 2007), and transfected using Lipofectamine 2000 (Invitrogen) 2d after plating. Cells were maintained 2d in culture before imaging. HEK-293 cells were obtained from ATCC and maintained in DMEM (Fisher Scientific) +10% FBS. Cells were transfected with Effectene (Qiagen). Stable cell lines were generated with Geneticin (Invitrogen) selection. Cells were passed to 25mm coverglass 1d after transfection and imaged the following day.

#### Immunofluorescence Ratiometric Recycling Assay and Quantification

TGs expressing FLAG-MOR were labeled with Alexa 488 conjugated M1 anti-FLAG to label surface receptors for 10m, followed by incubation with 10 $\mu$ M DG (Sigma) for 20 m to promote receptor internalization. Agonist media was washed out and replaced with media containing 10  $\mu$ M naltrexone (Sigma), a MOR antagonist, to prevent additional activation and internalization of MOR for 20 m. Recycled surface M1anti-FLAG labeled receptors were then labeled with secondary goat anti-mouse conjugated to Alexa 568 for 10 m at 4°C. All other incubations were performed at 37°C.

Cells were then fixed with 4 % PFA for 20 m, and blocked with 0.1M glycine in complete PBS for 10 m. A surface control, where cells were labeled for 10 m with Alexa 488-M1 anti-FLAG, immediately followed by Alexa 568-secondary goat anti mouse to quantify steady state amount of surface receptors. An endocytosis control was performed, where cells were labeled with the secondary antibody and fixed, to quantify the amount of receptors internalized in the presence of DG. Percent recycling was calculated from the ratio of intensities of the secondary antibody to the primary anti-FLAG and dividing experimental conditions by the surface control minus the endocytosis control (expt condition-endo ctrl.)/ (surf ctrl-endo ctrl.)%. Just Another Colocalization Plugin (JACoP) for ImageJ was used to generate a cytofluorogram and Pearson's Correlation Coefficient of intensities between primary and secondary antibody fluorescence. Statistical analyses and graphing were done using Microsoft Excel and Graphpad Prism. P values are from Mann-Whitney tests.

#### Individual Exocytic Event Recycling Assay

HEK-293 cells stably expressing SpH-MOR were incubated in DG for 5 m, and a 1 m movie acquired at 10 Hz using TIRF-M, followed by subsequent incubation with the second drug and a 1 m movie, at 37°C. For SP experiments, cells were transiently transfected with HA-NK1R. Cells were incubated in anti-HA (Sigma), followed by Alexa-568 goat-anti mouse, both for 10 m. Cells were incubated for 5 m with DG, and a 1 m movie was acquired. SP was added for 5m, followed by a 1 m movie. Individual insertion events were manually counted using a double blind process. A paired comparison was made within the same cell, normalizing to the agonist only treatment. Significance was determined through Student's paired t-test.

#### Live Cell and Fluorescence Imaging

Cells were imaged using a Nikon TE-2000E inverted microscope with a 60X 1.49 NA TIRF objective, Andor Revolution XD spinning disk confocal system, and 488 and 568 nm solid-state lasers. Cells were imaged in Opti-MEM or Leibowitz's L15 medium (Gibco), 5% FBS, at 37°C. Time lapses were acquired using an Andor iXon+ EM-CCD camera using Andor IQ. Original 16-bit tiff files acquired directly from camera were used for image analysis.

#### Fluorescent Ligand Binding

TG neurons were plated on a clear-bottom black 96-well plate, for 2d. Cells were stimulated with DG for 15 m to induce internalization, followed by a 15 m DG washout with naltrexone to induce MOR recycling, at 37°C. Two parallel controls, no DG or naltrexone treatment and a DG only treatment, were performed. Cells were washed and labeled with 100 nM Derm594 (in cold PBS, Ca/Mg) at RT, then washed out two times. Fluorescence was recorded on a Tecan Safire II Plate Reader at (at 25°C). Derm594 was generously donated by Dr. John Williams, Vollum Institute.

#### UMB-3 Immunofluorescence staining

TG neurons, plated on coverglass, were treated either with DG (endocytosis ctrl), no drug (surf ctrl), or incubated with DG for 20 m, followed by agonist washout with antagonist and vehicle, chel, or PMA for 20 m (recycling). Cells were then fixed in 4% PFA for 25 m, blocked and permeabilized in PBS + Ca/Mg, FBS, and .01% Triton for 45 m. Cells were incubated with UMB-3 in PBS + Ca/Mg at 4 °C overnight, and labeled with Alexa-488 goat anti-rabbit secondary antibody, mounted and imaged.

#### cAMP Measurement

Assays were performed on HEK293 cells stably expressing MOR and cAMPGlo Sensor 20F (Promega), at 35°C with IBMX. Luminescence was continuously recorded using a Tecan Infinite M1000 Plate Reader. After 5 m of baseline, DG was added for 10 m to record the initial response and allow endocytosis, media was washed out and replaced with media with naltrexone and either PMA, chel, or vehicle for 20 m for recycling. A rechallenge with DG was used to measure resensitization of recycled MORs.

#### Voltage Sensitive Dye Measurement

For control KCI and DG experiments, TG neurons were labeled with DiBAC<sub>4</sub>(5) and imaged every 30 s. 80 mM KCI was added to depolarize TGs, and DG was added 5 m after KCI to activate endogenous MORs. Cells were incubated with 10  $\mu$ M DG for 15 m. Agonist was washed out and replaced with media and 10  $\mu$ M naltrexone and PMA or chel , and compared to naltrexone only washout. Antagonist was washed out, for 20 m (chel) or 10 m (PMA), and cells were labeled with DiBAC<sub>4</sub>(5). 5 m after KCI, cells were rechallenged with 10  $\mu$ M DG and imaged. Mean fluorescence was analyzed using imageJ, and statistical analyses and graphing were performed in Graphpad Prism.

#### Tail Immersion Assay

Subjects were male C57BL6/J mice, between 9-12 weeks old. Animals were group housed in a 14h-10h light–dark cycle, and food and water was available *ad libitum*. All experiments were in accordance with AALAC guidelines, and were approved by the Animal Care Committee at the University Of Illinois at Chicago. Thermal

nociception was determined using the warm water tail withdrawal assay. Animals were initially habituated to the test apparatus for 2 days before testing. On the test day, mice were lightly restrained in a conical restraint bag, and their tails were immersed (5 cm from the tip) into a 52.5°C water bath. Tail withdrawal latencies were determined, and a cut-off of 12 s was established. After 3 basal measurements, mice were injected with fentanyl (0.1 mg/kg, SC) or morphine (5 mg/kg, SC) and tested every 30 m for 4.5-6 h (Melief et al., 2010). At 120 m (fentanyl) or 210 m (morphine) mice were injected intrathecally with 5  $\mu$ l of substance P (10 ng) or 0.9% saline. Intrathecal injections were performed with a 30 gauge, 1/2-inch needle at the L4-5 lumbar interspace on lightly anesthetized mice. Tail twitch was used to confirm needle placement, and any mice that exhibited motor impairment following IT injection were excluded. Mice were injected 30 m later with a second injection of fentanyl (0.1 mg/kg, SC) or morphine (5 mg/kg, SC), and tested every 30 m until tail withdrawal latencies returned back to baseline responses.

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#### **Author Contributions**

S.L.B, A.L.S, and M.A.P. conceived the project and designed experiments. S.L.B. performed performed two-color ratiometric recycling assays in TG neurons, live cell SpH-MOR imaging experiments in HEK-293 cells, live cell experiments with voltage sensing dye and dermporphin-594, and immunofluorescence experiments with UMB-3 anti-MOR. A.L.S. performed live cell SpH-MOR imaging experiments in HEK-293 cells, and performed site-directed mutagenesis of SpH-MOR S363-T370A. D.J.S performed experiments with Promega cAMP luminescence sensor. A.A.P performed antinociception assays in mice. S.S. developed and provided UMB-3 anti-MOR. S.L.B, A.L.S, D.J.S, A.A.P, and M.A.P. analyzed and interpreted data. S.L.B., A.L.S., and M.A.P. wrote this manuscript, which was reviewed by all authors.



# Fig S4-1. SpH-MOR Single event recycling assays are unaffected by changes in biosynthetic trafficking and NK1R trafficking, related to Fig 2.

A) HEK293 cells were either pre-treated with 10µM cyclohexamide for 4h at 37°C or not pre-treated with cyclohexamide, and incubated with DAMGO. Number of recycling events per minute after 5 min DAMGO incubation are plotted (n = 52, no treatment, n = 28, + cyclohexamide, error bars are s.e.m. across cells). B) Number of recycling events per minute before and after 5 min SP incubation (no DAMGO pre-treatment) are plotted, from HEK293 cells co-expressing SpH-MOR and HA-NK1R (n = 20, error bars are s.e.m. across cells).



**Fig S4-2.** PKC inhibition or activation does not affect MOR recycling or signaling without the presence of agonist, related to Fig 3. A) Number of SpH-MOR recycling events per minute before and after 1 min chel addition in absence of DAMGO (n = 17 cells, error bars are s.e.m. across cells). B) Number of SpH-MOR recycling events per minute before and after 1 min PMA addition in absence of DAMGO (n = 6 cells, error bars are s.e.m. across cells). C). Luminescence-based cAMP detection assay, as in Fig 3B. Chel (green), PMA (blue), or vehicle (red) were added to cells before treatment with DAMGO. Chel and PMA pre-treatments did not affect the initial DAMGO challenge effect on cAMP levels (n = 3 separate experimental trials, error bars are s.e.m. across multiple trials.









**Fig S4-3. Design of thermal antinociception assay and paired responses from individual animals, related to Fig 7.** A) Schematic of warm-water tail withdrawal assay. Baseline measurements were taken, animals were injected with agonist (fentanyl shown), and tail-withdrawal latencies were measured every 30 min. Saline (vehicle ctrl) or SP was injected intrathecally after initial response returned to baseline (120 min for fentanyl fentanyl; 210 min for morphine). B) Graph of responses of individual mice to initial fentanyl injection and rechallenge for vehicle ctrl and SP animals. C) Graph of responses of individual mice to morphine injection and rechallenge for vehicle ctrl and SP injected animals.

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## **Conclusions and Future Directions**

#### Summary

Together, the data in this thesis identify a role for downstream signaling kinases in the regulation of GPCR endosomal sorting to control receptor function. Chapter 2 shows that signaling kinases mediate hierarchical sorting of GPCRs into functionally distinct endosomal microdomains. In the case of the prototypical GPCR, the beta-2 adrenergic receptor (B2AR), PKA regulates sorting of the receptor between endosomal ASRT domains and constitutive tubules. PKA-regulated sorting of B2AR also generates an endosomal signaling bias. B2ARs recruit active Go stimulatory proteins exclusively from endosomal ASRT domains, suggesting that these endosomal microdomains play a role in controlling GPCR signaling, as well as sorting. PKA controlled sorting of B2AR created an endosomal signaling bias at the level of gene products downstream of endosomal cAMP production, suggesting that hierarchical sorting of GPCRs modulates receptor activity at the level of endosomal signaling.

Chapter 3 of this thesis addresses the physiological relevance of GPCR hierarchical sorting by testing the role of PKC regulation of mu-opioid receptor (MOR) endosomal sorting and endosomal signaling. PKC regulates MOR's entry into an actindependent recycling pathway via MOR PKC sites, S363 and T370. MOR S363 controls MOR sorting to Rab4 and Rab11 endosomal compartments. The drug selective phosphorylation site, T370, regulates MOR sorting into actin-stabilized tubules on endosomes. Further, phosphorylation at T370 may regulate a functional endosomal signaling bias of MOR, but further investigation of the endosomal signaling profile of MOR and the Ga inhibitory protein is needed. Together, Chapter 3 suggests that

GPCRs can undergo agonist-selective regulation via hierarchical endosomal sorting regulated by signaling kinases.

Chapter 4 of this thesis tests whether GPCR hierarchical sorting is subject to control by heterologous signaling pathways within physiologically relevant cell types. Together, our data show that MOR recycling to the cell surface is increased by pain signaling through activation of the neurokinin-1 receptor (NK1R) by substance P (SP). PKC was both required and sufficient for this increase in MOR recycling. SP-regulated MOR recycling requires PKC phosphorylation of MOR at S363 and T370. Further, our results show that SP regulates resensitization to opioid signaling in sensory neurons and antinociception in mice. Interestingly, SP and PKC regulated DAMGO- and fentanyl-mediated MOR recycling and resensitization, but not morphine-mediated MOR activity. Together, Chapter 4 suggests that pain signaling can heterologously regulate opioid action by controlling membrane trafficking of MOR, and that this regulation is agonist-selective.

#### Investigating the mechanistic basis of GPCR hierarchical sorting

This thesis shows that hierarchical sorting of GPCRs by downstream signaling kinases controls GPCR activity by regulating both the kinetics of receptor recycling and resensitization to cell surface signaling, and by controlling initiation of endosomal G protein signaling. The mechanism underlying how signaling kinase phosphorylation of GPCRs remains unknown. Understanding the molecular mechanisms of how signaling kinases change GPCR sorting may provide new insights into development of novel GPCR therapeutics that are specific to interactions with hierarchical sorting machinery for different GPCRs.

Ongoing work in our laboratory is aimed at identifying the mechanism of GPCR hierarchical sorting by investigating how the B2AR is restricted to endosomal ASRT domains. Stable Isotope Labeling of Amino acids in Cell Culture (SILAC) and mass spectrometry was performed on cells expressing B2AR to identify endosomal binding partner candidates of B2AR enriched after incubation with the agonist, isoproterenol (unpublished data, Rachel Vistein, in collaboration with Dr. Victor Faundez's laboratory, Emory University). Several candidates were identified, and our lab has begun testing the role of these candidates in B2AR recycling and differential binding to wild type B2AR and B2AR with the PKA-regulated hierarchical sorting sites, S345 and 346 mutated to alanine (SS>AA). We hypothesize that an endosomal protein binds to wild type B2AR, and not SS>AA, to restrict the receptor to endosomal ASRT domains, and that knockdown of this binding partner will sort B2AR into constitutive recycling tubules and increase recycling kinetics. Preliminary results suggest that knockdown of vacuolar sorting protein 8 (Vps8), an endosomal protein, increases B2AR recycling. Ongoing work will test if Vps8 binds preferentially to wild type B2AR over SS>AA and if it plays a role in B2AR signaling. An additional interesting future direction will be to investigate if endosomal machinery that controls B2AR hierarchical sorting is a conserved mechanism for other GPCRs. For example, this thesis shows that MOR undergoes hierarchical sorting via PKC, but not PKA, phosphorylation. A preliminary SILAC, mass spectrometry profile of MOR, identified several candidates that are distinct from those in the B2AR SILAC results, suggesting that the regulation of B2AR and MOR hierarchical sorting may be distinct. It will be interesting to test if MOR hierarchical sorting is

regulated by an independent mechanism from that of B2AR, and if these machinery control agonist-selective MOR endosomal sorting.

#### The functional consequences of GPCR sorting to distinct endosomes

Chapter 3 of this thesis suggests that hierarchical sorting of MOR by PKC phosphorylation at S363 sorts the receptor into distinct endosome populations. The exact endosomal compartment that S363A localizes MOR to, or the physiological relevance of this compartment, is not yet known. Current work is ongoing to identify the endosomal compartment that MOR-S363A localizes to and if the wild type receptor localizes to this subpopulation of endosomes. Interestingly, our collaboration with Dr. Aylin Hanyaloglu and colleagues at Imperial College London identified a GPCR endosomal compartment upstream of EEA1/Rab4 early endosomes (Jean-Alphonse et al., 2014). This work showed that the luteinizing hormone receptor (LHR) trafficked to a pre-early endosome (EE) compartment marked by the adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1 (APPL1). LHR's localization to pre-EEs requires an interaction of its C-terminal tail with the PDZ protein, GIPC (Jean-Alphonse et al., 2014). LHR did not colocalize with early endosomal markers, and recycled to the plasma membrane from pre-EEs. The pre-EE endosomes that LHR localizes to are a smaller population of endosomes than the EEs that the B2AR localizes too. Interestingly, sorting of LHR to pre-EEs was also required for initiation of MAPK signaling through LHR (Jean-Alphonse et al., 2014), suggesting that distinct populations of endosomes can be scaffolds for different types of GPCR signaling cascades.

Chapter 2 of thesis suggests that MOR may localize both to an EE compartment and a smaller population of endosomes, but it unknown if this is the same pre-EE compartment that LHR traffics to. Future work is needed to fully characterize the population of MOR endosomes. An interesting future direction will be to determine if different populations of MOR endosomes can initiate diverse signaling cascades, for example, through the MAPK pathway at pre-EEs and through a G protein pathway at EEs. Sorting of GPCRs to physically separate intracellular signaling compartments could provide the cell with a way to initiate different cellular responses by changing membrane trafficking of receptors. Further, it is unknown which endosomal compartments most GPCRs traffic to in physiological relevant cell types. An interesting future direction will be to study the subcellular compartmentalization of GPCRs in polarized cells, like neurons.

#### Physiological relevance of hierarchical sorting of GPCRs by signaling kinases

This thesis suggests that hierarchical sorting of GPCRs by signaling kinases can control if, and what type of signal is generated by a receptor. Chapter 4 shows that PKC-regulated trafficking of MOR is agonist selective, and preliminary work in Chapter 3 suggests that the agonist-selective phosphorylation site, T370, could potentially control downstream targets of endosomal G protein signaling. *I hypothesize that agonist-selective hierarchical sorting of MOR controls initiation of MOR endosomal G protein signaling*. Future work is necessary to determine if MOR activates the inhibitory Ga protein (Ga<sub>i</sub>) from endosomal membranes. Currently, I am performing additional quantitative, real-time PCR experiments with MOR and MOR-T370A to ask if the MOR-T370A mediated decrease in expression of the endosomal cAMP-dependent gene,

PCK1, requires activity of Ga<sub>i</sub> by asking if this effect on PCK1 is pertussis-toxin sensitive. Further, I am testing whether endosomal actin domains are required for this effect using latrunculin A treatment of MOR-T370A cells. To ask if MOR inhibits adenylyl cyclase from endosomal membranes, I am generating an endosomal targeted EPAC cAMP FRET sensor. Other potential future directions include imaging a fluorescently tagged Ga<sub>i</sub> to determine its endosomal localization and generating a nanobody biosensor that recognizes the Ga<sub>i</sub> active conformation to test which subcellular compartments MOR activates its G protein from.

The agonist selective nature of MOR-T370, and its potential for regulating MOR G protein signaling, raises the interesting possibility that different opioid agonists could control the spatial encoding of G protein signaling in a cell to produce diverse cell responses. I have recently attempted to perform quantitative real-time PCR of PCK1 in neurons with different agonists, but further optimization of this method in neuronal cells is ongoing. *I hypothesize that morphine will inhibit expression of the endosomal cAMP-dependent gene, PCK1, like MOR-T370A, but that agonists that induce phosphorylation at T370, like DAMGO, will not.* Agonist selective regulation of endosomal signaling suggests a mechanism for how different opiates, for example opiate drugs versus endogenous opioid peptides, could produce diverse long lasting effects and consequences.

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