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Effects of G Protein Signalling Modulator 3 on Cellular Signalling

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Abstract

G protein coupled receptors (GPCRs) promote G protein heterotrimer (Gα•GDP/Gβγ) activation. GPCR signalling is limited via G protein GTPase activity and β-arrestin-receptor interactions. G Protein Signalling Modulators (GPSMs) are proteins that may influence receptor signalling through G protein activity. GPSM3 modulates their activity by binding to $G\alpha_i$-GDP, limiting nucleotide exchange and preventing its re-association to Gβγ. The impact of GPSM3 on signalling is unknown. I hypothesize that GPSM3 will decrease $G\alpha_i$-dependent signalling while promoting Gβγ-dependent signalling in G_i-coupled GPCRs.

GPSM3 significantly inhibited β-arrestin recruitment to α_{2A}-adrenergic and μ-opioid receptors via a Gβγ-dependent mechanism, with no effect to G_s- and $G_{q/11}$-coupled GPCRs. N-terminal truncation and single point mutations in three distinct regions of GPSM3 decreased the inhibitory effect of GPSM3 on β-arrestin recruitment to α_{2A}-adrenergic and μ-opioid receptors.

Thus, my data suggest that GPSM3 negatively regulates β-arrestin-G_i-coupled GPCR interactions, which could serve as a potential therapeutic target for future pharmaceuticals.

Keywords

G Protein-coupled receptor (GPCR), G Protein Signalling Modulator 3 (GPSM3), cyclic 5'-monophosphate (cAMP), inhibition, β-arrestin, adenylyl cyclase, complementation-based bioluminescence, selective agonist, human embryonic kidney cells, pharmacology

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List of Abbreviations

AC	Adenylyl Cyclase
AGS	Activator of G Protein Signalling
βAR	β-adrenergic Receptor
βARK	β-adrenergic Receptor Kinase
BRET	Bioluminescence Resonance Energy Transfer
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CCPA	2-Chloro-N(6)-cyclopentyladenosine
CO_2	Carbon Dioxide
CTX	Cholera Toxin
DAG	Diacylglycerol
DAMGO	[D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin
DMEM	Dulbecco's Modified Eagle's Medium
DMB-FSK	7-Deacetyl-7-[O-(N-methylpiperazino)-γ-butyryl]-Forskolin Dihydrochloride
DMSO	Dimethyl Sulfoxide
EN	Epinephrine
EPAC	Exchange Protein directly Activated by cAMP
FBS	Fetal Bovine Serum

GABA	Gamma-Aminobutyric Acid
GAP	GTPase Activating Protein
GDI	Guanine Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GIP	G Protein Coupled Receptor Interacting Protein
GTP	Guanosine Triphosphate
GPCR	G Protein Coupled Receptor
GPR	G Protein Regulatory
GPSM3	G Protein Signalling Modulator 3
GRK	G Protein Receptor Kinase
HEK	Human Embryonic Kidney
HGNC	Human Gene Nomenclature Committee
HUGO	Human Genome Organization
IBMX	3-isobutyl-1-methylxanthine
IP_3	Inositol-1,4,5-triphosphate
LGN	Leucine-Glycine-Asparagine
M1	Muscarinic Type 1
MEK	Mitogen-activated Protein Kinase Kinase
MEM	Minimum Essential Medium

O_2	Oxygen
PBS	Phosphate Buffered Saline
PH	Pleckstrin Homology Domain
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLCβ	Phospholipase C-β
PKA	Protein Kinase A
PKC	Protein Kinase C
PTH1R	Parathyroid Hormone Type 1
PTX	Pertussis Toxin
RBD	Ras-binding Domain
RLuc	*Renilla* Luciferase
RGS	Regulator of G Protein Signalling
RLU	Relative Luminescent Units
RH	Regulator of G-protein Signalling Homology Domain
TPR	Tetratrico-peptide Repeat
UK 14,304	5-Bromo-6-(2-imidazolin-2-ylamino)-quinoxaline tartrate
YFP	Yellow Fluorescent Protein

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Chapter 1

1.1 INTRODUCTION

1.1.1 *DRUG-RECEPTOR INTERACTIONS*

Many drugs exert their effects by binding to specific receptors within the human body. Drug efficacy is often proportionally related to the amount of a drug at the site of action and can be modeled by examining the relationship between drug concentration and its overall effect (Hryniuk *et al.* 1990). Furthermore, these effects can be quantified using parameters like molecular efficacy and potency (Figure 1-1) Molecular efficacy refers to the ability of an agonist, once bound to a receptor, to initiate a maximal response or effect (E_{max}), whereas potency is reflected in the concentration of drug that simulates (EC_{50}) or inhibits (IC_{50}) the maximum effect by 50% and correlates inversely with these parameters (*i.e.* high potency drugs produce their effects at low concentrations) (Luk *et al.* 2009, Kim *et al.* 2018) . The continued use of an agonist may reduce its effectiveness over time. This phenomenon is referred to as tolerance and may be influenced by pharmacokinetic factors, especially metabolism, which may decrease the overall bioavailability of the drug at the site of action (Ferguson *et al.* 2001, Marchant *et al.* 1981). Alternatively, decreases in drug efficacy and/or potency may also occur via changes in the receptor involved, its signalling partner proteins, or upstream or downstream components of the signalling pathway that mediates the effects of the drug. Finally, *in vivo* responsiveness to the effectiveness of a drug can be muted by the activation of physiological processes that counter its effects. Overall, clinicians need to be aware of pharmacokinetic, pharmacodynamics and physiological factors in order to prescribe and maintain optimal

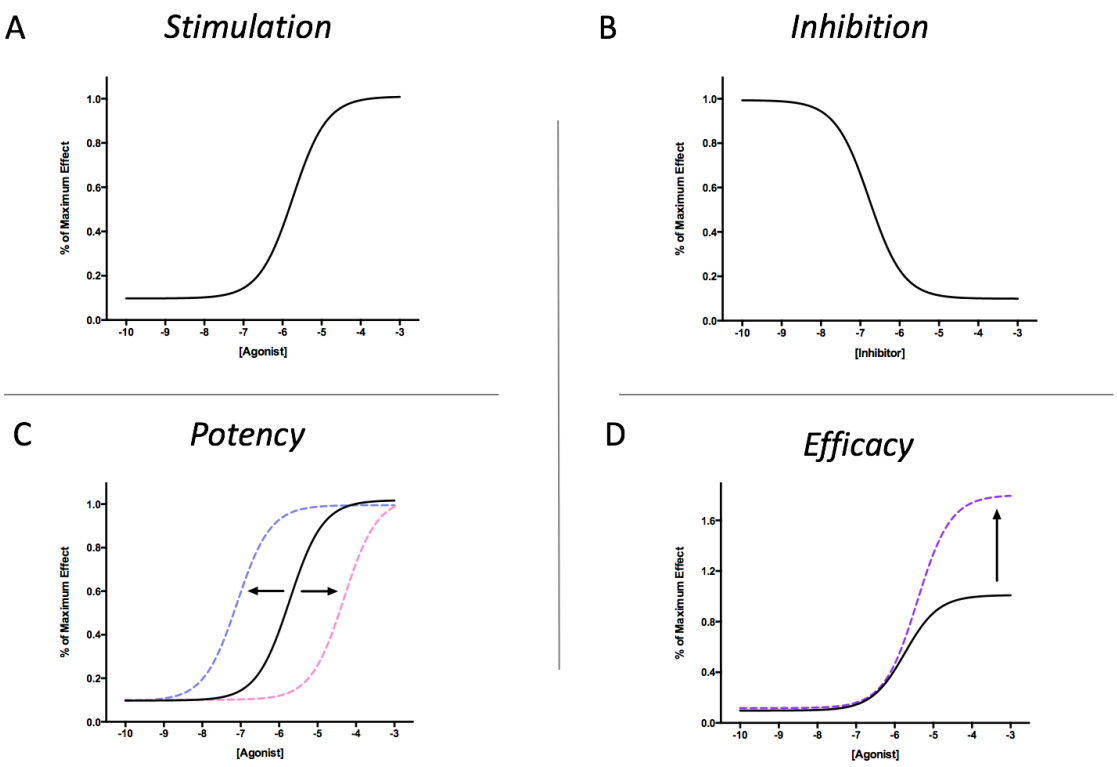

Figure 1-1 Effects of ligand activation on G Protein Coupled Receptor activity. Ligand binding can lead to either *stimulation* (A) or *inhibition* (B) of receptor activity. Moreover, activation of a receptor can also lead to a negative response (*e.g.* inhibition of cAMP production by G_i-coupled GPCR activation) (B). Furthermore, different ligands can modulate receptor effects by exhibiting different binding affinities to the receptor, which in turn alters agonist *potency* (C) but can also alter overall receptor activity or *efficacy* (D).

drug therapies that will maximize drug efficacy while minimizing potential toxicity and adverse effects.

1.2 G PROTEIN COUPLED RECEPTORS

1.2.1 *OVERVIEW*

G-protein coupled receptors (GPCR) are the largest family of cell surface receptors encoded by the mammalian genome and play a wide range of important roles in physiological processes, such as metabolism, endocrine function, olfaction, vision, neuromuscular regulation and central nervous system function (Duc *et al.* 2015). As a result, GPCRs are widely exploited as targets for drugs to treat many diseases. Over 30% of current pharmaceuticals modulate GPCR activity by mimicking or inhibiting endogenous ligand activity (Santos *et al.* 2017, Rask-Anderson *et al.* 2014, Hauser *et al.* 2017).

GPCRs are heptahelical structures predominantly expressed in the plasma membranes of eukaryotes, including yeast and mammals. Approximately 800 GPCRs that have been identified in humans with different sensory and signalling functions (McCudden *et al.* 2005, Palczewski *et al.* 2006). Structurally, GPCRs are comprised of a single polypeptide chain with an extracellular amino terminus, seven hydrophobic alpha-helical transmembrane domains and an intracellular carboxyl terminus. The seven transmembrane domains are held together by three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3) (Zhang *et al.* 2015). The N terminus, ECL1-3, and usually a pocket formed by the outer halves of the transmembrane domains are

collectively responsible for ligand recognition, binding and modulation of ligand access to the extracellular surface of the receptor. Two cysteine residues found on the extracellular loops of GPCRs, specifically Class A receptors (discussed in the next section), are highly conserved between species and confer stability when oxidized to form disulfide bonds (Tuteja *et al.* 2009). Intracellular loops are vital for modulation of GPCR signalling. The cytoplasmic domains contain serine and threonine residues, which can be phosphorylated by G protein receptor kinases (GRKs). This phosphorylation initiates a sequence of events, which may desensitize the receptor from further agonist stimulation and target the GPCR for degradation, recycling or endosomal signalling.

GPCRs are activated by a multitude of ligands including hormones, neurotransmitters, photons, ions, chemokines, lipids and other molecules that can range in size from small molecules to peptides and even large glycoproteins (Tyndall *et al.* 2005). Evidence shows that various GPCRs may also be responsive to mechanical stimuli, such as fluid shear stress, tension and compression (Dela Paz *et al.* 2017, Scholz *et al.* 2016). GPCRs have strong affinities for specific ligands making them desirable drug targets (Schoneberg *et al.* 2004). While many GPCRs have been identified and paired up with their biological activators, there are still approximately 100 of these receptors (named orphan receptors) for which no endogenous ligand has been confirmed (Atwood *et al.* 2011).

1.2.2 *CLASSIFICATION OF G PROTEIN-COUPLED RECEPTORS*

Based on the structural and sequence similarities among receptors, the GPCR superfamily can be divided into six different families: Family A, rhodopsin-like; Family B, secretin-like; Family C, metabotropic glutamate; Family D, fungal pheromone receptor; Family E, *Dictyostelium* cAMP receptor; and Family F, Frizzled/Smoothened and lastly, Adhesion receptors (Davies *et al.* 2007, Hu *et al.* 2017, Horn *et al.* 2003). Family A is the largest group, comprising nearly 85% of the known GPCR-encoded genes. This family largely encodes for a variety of small molecule (*e.g.* acetylcholine, noradrenaline), peptide (*e.g.* angiotensin, endothelin), glycoprotein (*e.g.* follicle stimulating hormone) and olfactory receptors, which exhibit sequence homology to rhodopsin (Atwood *et al.* 2011). On the other hand, there are 15 Family B1 secretin-like receptor genes and many family B2 adhesion receptors genes encoded within the human genome. The secretin-like receptors respond to peptide ligands that are structurally similar to each other. Furthermore, Family C GPCRs consist of GABA, calcium-sensing receptors as well as several taste receptors abundantly present in rodents but absent in humans. Family D and E are largely absent in vertebrates, while family F receptors predominantly signal independently of heterotrimeric G proteins (Attwood *et al.* 1994, Bjarnadottir *et al.* 2006, Fredriksson *et al.* 2003). Despite the large variety of GPCRs known today, only a fraction of these receptor have been studied as therapeutic agents.

1.2.3 *G PROTEIN-COUPLED RECEPTOR SIGNALLING*

GPCRs are bound to intracellular heterotrimeric G Proteins (Gα•GDP/Gβγ) in their inactive state. Activation typically occurs when an agonist binds to the extracellular and/or transmembrane region of the GPCR and induces a conformational change in the receptor. This change causes the receptor to function as a guanine nucleotide exchange factor (GEF) to promote the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) at the Gα subunit (Hepler *et al.* 1992, McCudden *et al.* 2005). The Gα•GTP/Gβγ complex subsequently is thought to dissociate into Gα•GTP and Gβγ dimer, which then go on to activate their respective downstream signalling pathways (Figure 1-2) (Wall *et al.* 1998). Signalling persists until GTP is hydrolyzed back to GDP by the Gα subunit, which has intrinsic GTPase function (Hepler *et al.* 1992). In G proteins other than G_s, this GTPase function can be accelerated by Regulators of G Protein Signalling (RGS) and other GTPase Activating Proteins (GAPs), which are further discussed in section 1.2.7. As a result, the GDP-bound Gα subunit re-associates with the Gβγ dimer thereby returning the heterotrimer to its inactive state (Gilman *et al.* 1987).

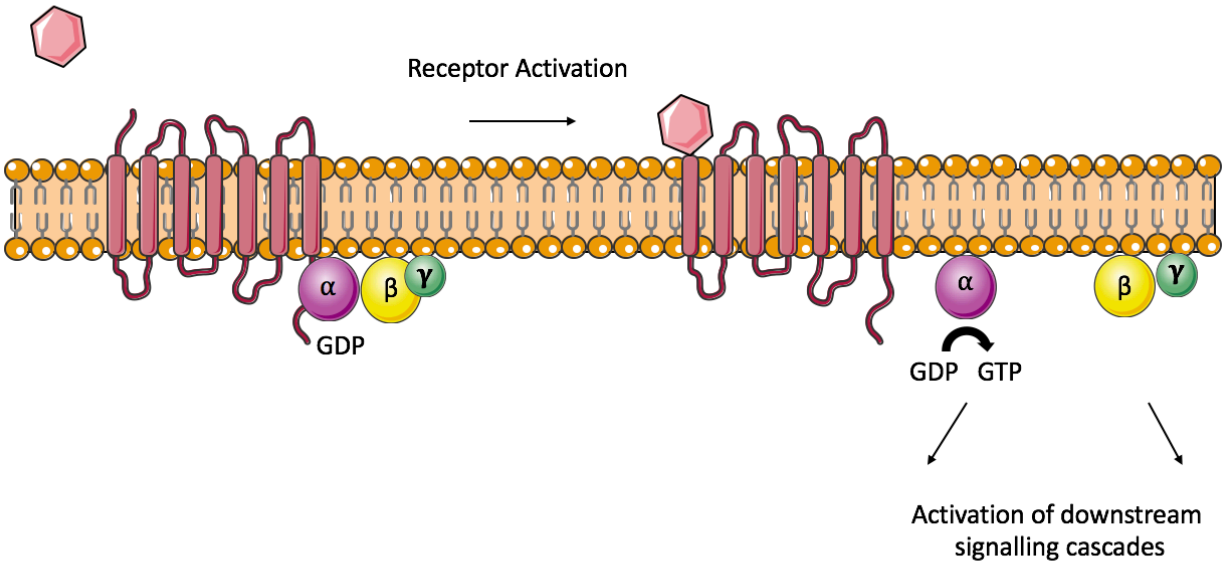

Figure 1-2. G protein-coupled receptor activation. Schematic diagram represents the process of receptor activation. Agonist binding to the G protein-coupled receptor induces a conformational change that stimulates guanine nucleotide exchange factor (GEF) activity within the receptor. This activity then promotes the exchange of GDP for GTP at the Gα subunit. Consequently, Gα and Gβγ subunits dissociate from the complex and activate their respective downstream signalling processes.

1.2.4 *G PROTEIN DIVERSITY*

As of today, 23 Gα, 7 Gβ and 12 Gγ subunit isoforms have been identified (Smrcka *et al.* 2015, Khan *et al.* 2013). Multiple combinations of isoforms can exist in any given cell type (Brann *et al.* 1987, Betty *et al.* 1998, Straiker *et al.* 2002). Despite the variety of G protein isoforms, there are three main G protein-dependent signalling pathways mediated by four members of Gα subunits: $G\alpha_{i/o}$, $G\alpha_{s}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Figure 1-3, Bockaert *et al.* 2003). Each subtype has different modulatory properties on downstream effectors, despite being activated by a similar mechanism. The downstream effector shared by both $G\alpha_{i/o}$ and $G\alpha_{s}$ subclasses is adenylyl cyclase, which is an enzyme responsible for the conversion of adenosine triphosphate (ATP) to 3',5'-cyclic adenosine monophosphate (cAMP) and pyrophosphate. The $G\alpha_{i/o}$ family ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o}$, $G\alpha_{z}$, $G\alpha_{t}$) directly inhibits adenylyl cyclase from producing cAMP, whereas $G\alpha_{s}$ (Gαs, $G\alpha_{olf}$) directly stimulates cAMP production. Cyclic AMP is an essential second messenger that is capable of activating further downstream targets, such as activation of cyclic nucleotide-gated ion channels and members of the protein kinase A family (Weinstein *et al.* 2004), as well as the small G protein exchange factor called Exchange Protein directly Activated by cAMP (EPAC) (Jeevaratnam *et al.* 2018, Xiao *et al.* 2018). On the other hand, the $G\alpha_{q/11}$ subunit ($G\alpha_{q}$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, $G\alpha_{16}$) activates the effector phospholipase C-β (PLCβ) that initiates the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Both PIP_2 and DAG serve as second messengers to stimulate calcium release from the endoplasmic reticulum and activate some isoforms of protein kinase C (PKC), respectively. Additionally, the $G\alpha_{q/11}$ subunit can also activate the effector protein Rho via p64RhoGEF (Kamato *et al.* 2015). Similarly, the $G\alpha_{12/13}$ subclass can activate

three RhoGEF subtypes: PDZ-RhoGEF, p115-RhoGEF and LARG. The $G\alpha_{12/13}$-RhoGEF complex activates small GTPase, Rho, in an allosteric manner, which serves as a second messenger to activate various downstream proteins, often involved in cytoskeletal regulation.

The other important heterotrimeric G protein component in signalling cascades is the Gβγ subunit, which functions as a dimer. The complex is composed of a tight association between one Gβ and one Gγ subunit and it collectively dissociates from the Gα subunit shortly after receptor stimulation. Prior to G protein activation, the inactive GDP-bound form of Gα is stabilized by the binding of Gβγ to the heterotrimeric complex (Brandt *et al.* 1985). Gβγ signalling is diverse and functional activity depends on the interaction of different Gβγ isoforms with different effector proteins. One example of the established effects of Gβγ signalling is through its interactions with different isoforms of adenylyl cyclases (Table 1.1) (Tang *et al.* 1992, Sunahata *et al.* 2002, Sabbatini *et al.* 2016). Furthermore, the Gβγ dimer also modulates the activities of voltage-dependent Ca^{2+} channels, phospholipase C production and phosphatidylinositol-3-kinase activity (Lotersztajn *et al.* 1992, Stephens *et al.* 1994). In addition, the Gβγ dimer serves as a negative regulator of Gα when it is bound to it by increasing the affinity of Gα for GDP and has also been shown to foster interactions of Gα with appropriate receptors (Im *et al.* 1988, Kisselev *et al.* 1994). Notwithstanding this inhibitory effect on nucleotide exchange, Gβγ is required for GPCR-promoted nucleotide exchange on Gα (Oldham *et al.* 2006, Gilman *et al.* 1987).

Heterotrimeric G proteins are naturally found anchored to the plasma membrane via post-translational lipid modifications on cysteine residues that target these proteins to the plasma membrane for signal transduction (Gilman *et al.* 1987, Casey *et al.* 1994). There are three types of lipid modifications: palmitoylation, myristoylation and prenylation (Kinsella *et al.* 1994). Palmitoylation and myristiolation occur predominantly on Gα subunits near the amino terminus and are essential for appropriate coupling of Gα subunits to their respective effectors (Linder *et al.* 1993, Parenti *et al.* 1993). The γ subunit undergoes prenylation and carboxylmethylation shortly after binding to the Gβ subunit. These modifications are essential for facilitating interactions between the Gβγ subunit with Gα proteins, the receptor but also effectors (Wedegaertner *et al.* 1995, Lindorfer *et al.* 1996). Interestingly, the β subunit is not subject to lipid modifications; however, it remains anchored to the plasma membrane via binding interactions with the γ subunit (Casey *et al.* 1994).

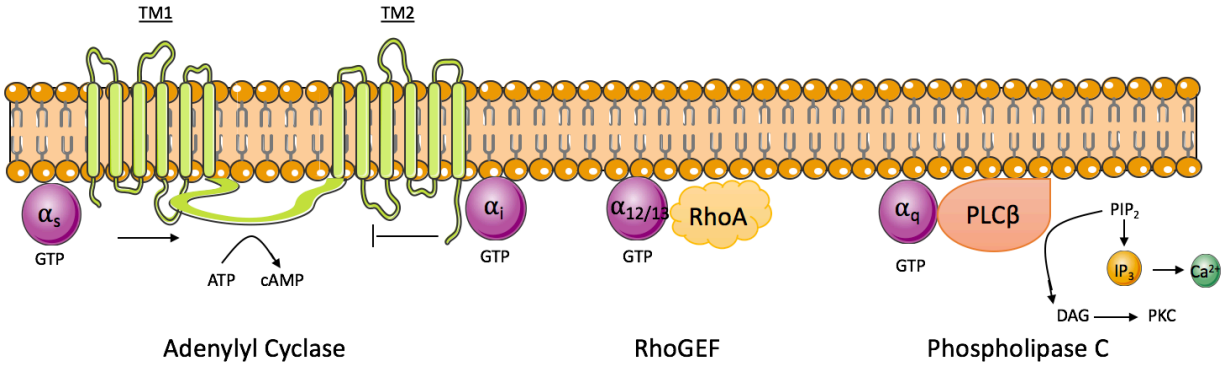

Figure 1-3. G-protein dependent signalling. Ligand-induced GPCR activation promotes nucleotide exchange and subsequent dissociation of G protein heterotrimers (Gα•Gβγ) into free Gα and Gβγ subunits. These subunits can then go on to activate their downstream signalling pathways. Gα-mediated signalling can be divided accordingly to protein subtype: i) $G\alpha_s$ stimulates the activity of effector adenylyl cyclase, which catalyzes the conversion of ATP to cyclic 5'-adenosine monophosphate (cAMP), ii) $G\alpha_i$ inhibits adenylyl cyclase, iii) $G\alpha_{q/11}$ activates the enzyme phospholipase Cβ, which catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), which promotes the release of Ca^{2+} from the endoplasmic reticulum and activates the protein kinase C (PKC), respectively, and iv) $G\alpha_{12/13}$ promotes the nucleotide exchange at RhoA by activating RhoGEF.

Table 1.1. Regulatory properties of different isoforms of adenylyl cyclases

AC Isoforms	$G\alpha_i$	$G\alpha_s$	$G\beta\gamma$	Forskolin	Protein Kinase
Group I:					
ACI	-	+	-	+	(+, PKCα)
ACIII	-	+	-	+	(+, PKCα)
ACVIII	-	+	-	+	=
Group II:					
ACII	=	+	+	+	(+, PKCα)
ACIV	=	+	+	+	(+, PKC),(-, PKCα)
ACVII	=	+	+	+	(+, PKCδ)
Group III:					
ACV	-	+	+	+	(-, PKAτ),(+,PKCα/ζ)
ACVI	-	+	+	+	(-,PKAτ, PKCδ, ε)
Group IV:					
ACIX	-	+	=	Weak + or =	(-, novel PKC)
ACX	=	=	=	=	=

(-): inhibition of AC; (+): stimulation of AC; (=): no effect on AC

Adapted from Sunahara *et al.* 2002

1.2.5 *RECEPTOR-DEPENDENT REGULATION OF GPCR SIGNALLING*

GPCR signalling is regulated through many different mechanisms, one of which is the process of receptor internalization. Upon ligand activation, the receptor is phosphorylated by G protein-coupled receptor kinases (GRKs), which initiates a series of signalling cascades that may result in signal termination (Fig. 1.4). GRKs phosphorylate serine and threonine residues located on the third intracellular loop and/or cytoplasmic tail of the GPCR (Tobin *et al.* 2008). Phosphorylation promotes the recruitment and binding affinity of a family of scaffolding proteins called β-arrestins, which bind to the second and third intracellular loops but also the C-terminal tail of the GPCR (Kohout *et al.* 2003, Luttrell *et al.* 2002). Interactions between receptor and β-arrestin facilitate recruitment of additional adaptor proteins, such as clathrin and assembly protein complex 2 (Gaidarov *et al.* 1999, Goodman *et al.* 1996). Furthermore, GPCR-β-arrestin interactions quench G protein signalling by sterically inhibiting further G protein activation and ultimately, their re-association to the receptor (Szczepek *et al.* 2014). As a result, this turns off activity of G protein-dependent secondary messenger pathways.

The discovery of GRK proteins stemmed from studies performed in the 1970s and 1980s that focused on understanding the mechanisms behind the loss of rhodopsin and β-adrenergic receptor (βAR) signalling after prolonged stimulation. Studies have shown that prolonged agonist stimulation resulted in a mobility shift change along SDS PAGE, when compared with an unstimulated β-adrenergic receptor (Stadel *et al.* 1982). This shift in mobility was later attributed to the addition of multiple phosphate groups along the C-terminal region of the receptor (Stadel *et al.* 1983). Subsequent studies have established that this phosphorylation was directly related to the activity of protein kinases, such as cAMP dependent protein kinase

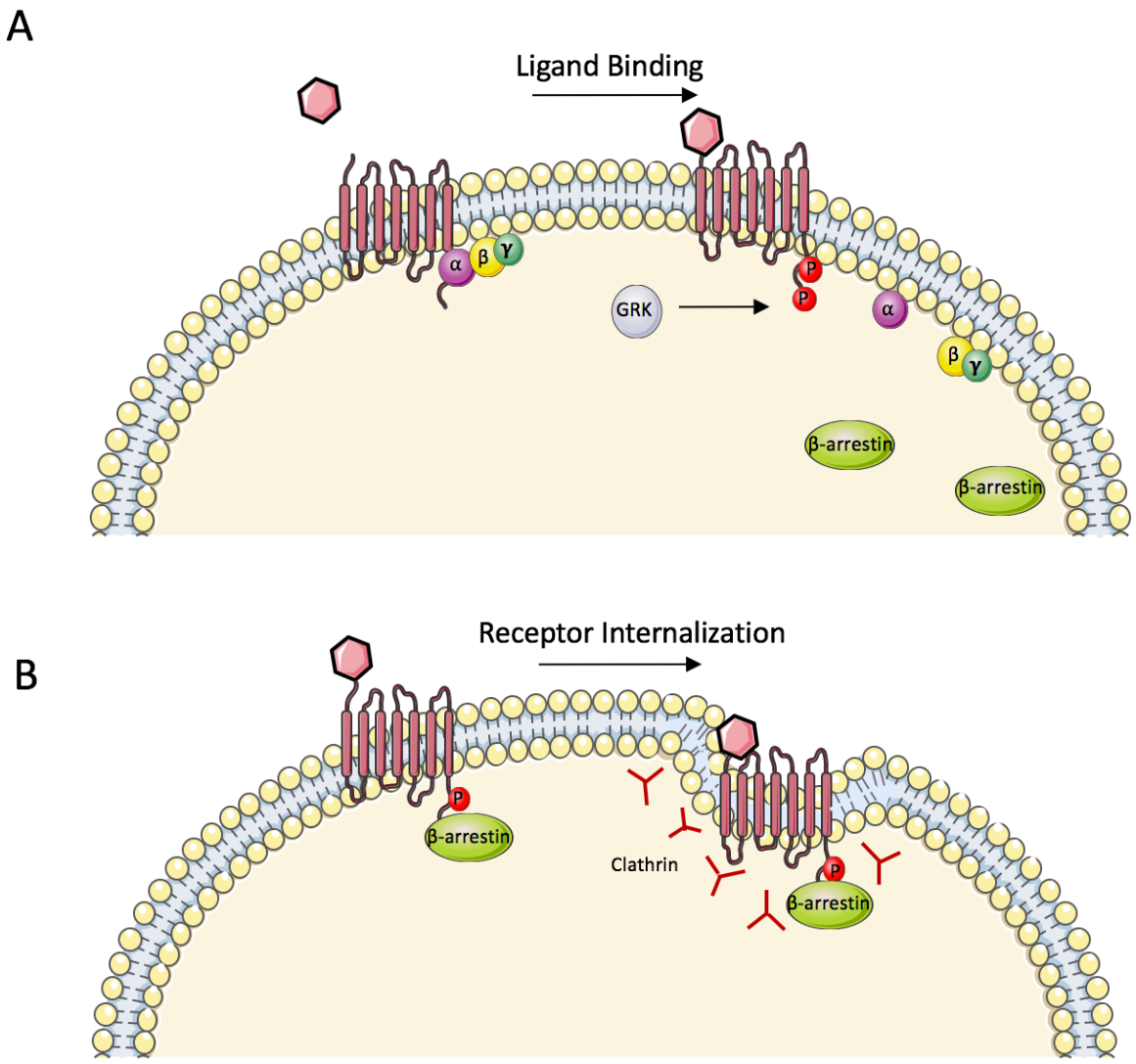

Figure 1-4. β-arrestin-mediated receptor internalization. (A) Shortly after agonist-induced receptor activation, serine and threonine residues on the C-terminal portions of the receptors are phosphorylated via G Protein Receptor Kinases (GRKs). (B) Phosphorylation increases the recruitment and binding affinity of β-arrestins to the receptor. Interactions between receptor and β-arrestin facilitates recruitment of additional adaptor proteins, such as clathrin and assembly protein complex 2, which target the receptor to clathrin-coated pits. This complex promotes internalization via endosomal formation.

called protein kinase A (PKA) and more commonly by GRKs, which facilitate the attenuation of receptor coupling to heterotrimeric G proteins (Sibley *et al.* 1984, Benovic *et al.* 1985). This phenomenon was eventually termed receptor desensitization and has been a useful tool in understanding receptor activity.

GRKs are protein kinases that specifically phosphorylate serine and threonine residues on agonist-occupied receptors. As of today, seven different GRKs have been identified: GRK1 (rhodopsin kinase), GRK2 (β-adrenergic receptor kinase-1 or βARK1), GRK3 (βARK2), GRK4 (IT-11), GRK5 (23,24), GRK6 (25) and GRK7. GRKs are classically grouped into one of three subfamilies based on similarities in sequence homology: visual (GRK1, GRK7), GRK2 (GRK2, GRK3) and GRK4 (GRK4, GRK5, GRK6) (Komolov *et al.* 2018). With the exception of the visual subfamily, GRKs are ubiquitously expressed within the human body (Pitcher *et al.* 1998). All members are comprised of two domains: a central catalytic domain and a Regulator of G-protein Signalling Homology (RH) domain. The catalytic core resides within the RH domain and is responsible for kinase activity. On the other hand, the RH domain facilitates interactions that promote the inactive conformation of the catalytic domain (Lodowski *et al.* 2006). GRKs are regulated via post-translational modifications on C-terminal regions, which differ between subfamilies. Visual GRKs are modified via prenylation whereas GRK2 and GRK4 subfamilies are modified via direct lipid binding through the pleckstrin homology (PH) domain and via palmitoylation, respectively (Komolov *et al.* 2018). Interestingly, there is evidence that certain GRKs could also interact with other proteins potentially implicated in receptor desensitization. Crystallography experiments have shown that some GRKs, specifically GRK2, can complex with $G\alpha_{q/11}$ and $G\beta\gamma$ (Tesmer *et al.* 2005).

As these studies continued, arrestin, a protein initially discovered in the visual system became identified as an attenuator of phototransduction. This led to an interest in identifying non-visual arrestin proteins capable of mediating desensitization. Studies by Lefkowitz and his team have discovered proteins with similar roles as the aforementioned arrestins in desensitizing β-adrenergic receptor signalling following phosphorylation by receptor kinases and as such, have called these proteins β-arrestins (Stadel *et al.* 1982, Stadel *et al.* 1983). Currently, there are four arrestins expressed in mammalian tissues: visual (arrestins 1 and 4) and non-visual (arrestins 2 and 3; β-arrestin-1 and -2, respectively) (Freedman *et al.* 1996, Luttrell *et al.* 2010).

β-arrestin proteins are ubiquitously expressed within mammalian cells and often exist in a constitutively phosphorylated state within the cytosol. β-arrestin-1 proteins can be phosphorylated by GRK5 and Erk1/2 kinases on residue Ser^{412} while β-arrestin-2 proteins are phosphorylated by casein kinase II on residues Thr^{276}, Ser^{361} and Thr^{383} (Lin *et al.* 1997, Cassier *et al.* 2017, Shenoy *et al.* 2011). The mechanism behind β-arrestin dephosphorylation remains elusive; however, it has been reported that protein phosphatase PP2A and Mitogen-activated protein kinase kinase (MEK) may play an important role (Xiao *et al.* 2007, Beaulieu *et al.* 2005, Shenoy *et al.* 2011). Phosphorylation of receptors by GRKs recruits β-arrestin proteins to these receptors at the plasma membrane. GRK-induced phosphorylation is an essential step in receptor desensitization and is also the rate-limiting step within the entire process. Upon binding, β-arrestin proteins become dephosphorylated and can initiate desensitization and subsequent internalization. Furthermore, β-arrestins can regulate a myriad of different signalling pathway, such as second messenger degradation, cytoskeletal rearrangement, transcriptional regulation via nuclear translocation and ubiquitination (Luttrell *et al.* 2002).

β-arrestin proteins target receptors to a clathrin-dependent endocytic pathway where receptors are internalized via endosomal formation. Once internalized, the receptor can be sorted to recycling endosomes, which shuttle the receptor back to the plasma membrane, or to multivesicular late endosomes, which target these GPCRs for lysosomal degradation. In addition, internalized receptors can also engage in cell signaling by modulating activity of multiple effectors, such as adenylyl cyclase, from within the endosome (Fig.1.5) (Calebiro *et al.* 2009, Feinstein *et al.* 2012, Ferrandon *et al.* 2009).

Two patterns of β-arrestin recruitment have been characterized according to receptor class. Class A receptors exhibit weak and transient binding to β-arrestin proteins after ligand activation. Thus, they are rapidly recycled back to the plasma membrane after they have been internalized. On the other hand, Class B receptors exhibit strong and prolonged binding to β-arrestin proteins and as a result, are recycled back to the plasma membrane more slowly after internalization (Thomsen *et al.* 2016, Luttrell *et al.* 2002). Class A receptors include but are not limited to α_{2A}-adrenergic, adenosine-A1, muscarinic type-1, β_2-adrenergic and μ-opioid, while Class B receptors include parathyroid hormone type-1 (Alexander *et al.* 2017).

1.2.6 *OTHER RECEPTOR INTERACTIONS THAT INFLUENCE SIGNALLING*

GPCR activity can be directly modulated through additional binding interactions with other receptors, agonists and GPCR-interacting proteins (GIPs) (Abramow-Newerly *et al,* 2006, Magalhaes *et al.* 2011). Receptor activity can be directly influenced by receptor-receptor interactions. These interactions can facilitate the formation of homo or hetero-oligomeric complexes, which can then change conformational states by increasing or decreasing affinity of

ligands to receptor sites (Chidiac, 2016). Furthermore, the extent of GPCR signalling can be affected by different classes of receptor-binding ligands, such as full, partial and inverse orthosteric agonists, as well as positive and negative allosteric modulators. Highly efficacious agonists exhibit strong preferences for binding to activated receptors, whereas weakly efficacious agonists have relatively less selectivity towards active as compared to inactive receptor conformations. Highly efficacious agonists, once bound, tend to prevent isomerization of the receptor back to its inactive state and thus, more readily initiate signalling (Onaran *et al.* 1997, Chidiac, 2016). Inverse agonists favour inactive conformations and thus promote a response opposite to that of an agonist. Moreover, activating ligands can differ based on where they bind on the receptor. Orthosteric refers to the primary binding site on the receptor whereas, allosteric refers to a site different from the endogenous binding site (Wang *et al.* 2018, Kim *et al.* 2018).

Allosteric modulators alter receptor conformation, which can then alter ligand binding affinity, potency and efficacy of downstream signalling. Lastly, GIPs can alter GPCR conformational properties by directly binding to the receptors. GIP-induced conformational changes may result in: i) changing receptor affinities and therefore selectivity for different agonists and ii) alter G protein binding interactions with the receptor (Hay *et al.* 2006, Morfis *et al.* 2008).

As alluded to above, GPCRs can also spontaneously isomerize between inactive and active conformations. It is now widely accepted that these receptors can assume multiple different conformations and as such, can signal pleiotropically (Chidiac *et al.* 2016, Onaran *et al.* 1997, Kenakin *et al.* 2010, Park *et al.* 2012).

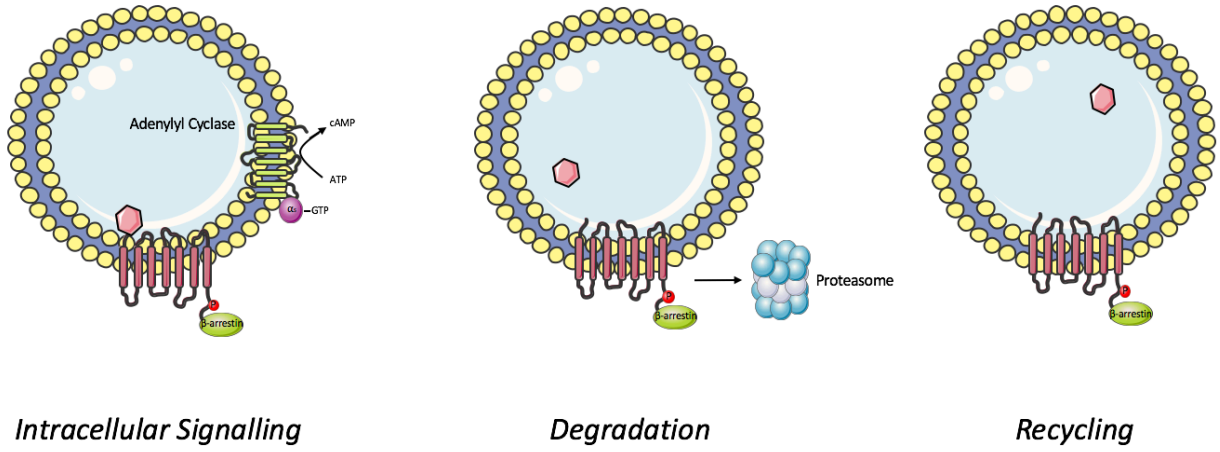

Figure 1-5. Fates of G protein-coupled receptor (GPCR) after endosomal formation. Within seconds of GPCR activation, G protein-coupled receptor kinases (GRKs) are recruited to the receptor where they phosphorylate the C-terminal domain. This phosphorylation initiates the recruitment of β-arrestin scaffolding proteins, which target the receptor for clathrin-coated pits. Subsequently receptors are internalized and can therefore: i) continue signalling from within the endosome, ii) become targeted for degradation via the ubiquitin proteasome pathways, and/or iii) become recycled back to the plasma membrane for additional signalling.

1.2.7 *RECEPTOR-INDEPENDENT REGULATION OF GPCR SIGNALLING*

GPCR activity can be directly regulated through G protein activation states. Heterotrimeric G proteins belong to a superfamily of GTPases. This superfamily also includes small signalling G proteins (*e.g.* Ras, Rho), initiation and elongation factors, signal recognition particle GTPases, dynamin and tubulin (Bourne *et al.* 1991, Daumke *et al.* 2016). These proteins have the intrinsic ability to self-deactivate by irreversibly hydrolyzing the γ phosphate of GTP into GDP and inorganic phosphate. In heterotrimeric G proteins, this process promotes the GDP-bound state while fostering its re-association to Gβγ. Thus, within the heterotrimeric complex, Gα functions as a GTPase to control the duration of activation. Additionally, the Gβγ dimer can also aid in GPCR signal termination by directly interacting with the 125-amino acid, carboxyl-terminal portion of GRK proteins, specifically GRK2 and GRK3 (Komolov *et al.* 2018). GRK recruitment to the plasma membrane is markedly enhanced and is dependent upon γ subunit isoprenylation (Pitcher *et al.* 1998). This physical interaction helps to enhance the rate of phosphorylation of agonist-activated receptors (Haga *et al.* 1990, Haga *et al.* 1992, Pitcher *et al.* 1992). Stoichiometric ratios between Gβγ and GRK are fundamental in initiating both the maximal rate and extent of enzymatic activity and deviations from optimal ratios may alter recruitment and enzymatic kinetics. One study has demonstrated that a molar ratio of 10:1 for Gβγ and GRK, respectively, significantly enhanced receptor phosphorylation when compared with absence of Gβγ; however, addition of Gα with free Gβγ has been shown to negate this effect of the latter (Pitcher *et al.* 1992).

GPCR signalling can be further modulated by three classes of G protein accessory proteins or G protein auxiliary proteins, called GTPase activating proteins (GAPs), guanine

nucleotide exchange factor (GEFs) and guanine nucleotide dissociation inhibitors (GDIs). These proteins are distinct from GPCRs, G proteins and classical effectors. G protein accessory proteins regulate the strength of G protein-mediated signalling by directly influencing G protein activity (Siderovski *et al.* 2005, Cismowski *et al.* 2006). These proteins can directly bind to G proteins in the presence or absence of receptor activation, regulate their activation state by altering nucleotide binding or hydrolysis properties, and some also can interfere with signal transfer between the receptor and respective G proteins (Sato *et al.* 2006) or between G proteins and effectors (Abramow-Newerly *et al.* 2006). As previously mentioned, Gα proteins have intrinsic GTPase activity which enables them to hydrolyze GTP into GDP in order to return back to the inactive state. This intrinsic GTPase activity can be markedly enhanced by GAPs (De Vries *et al.* 2000, Berman *et al.* 1996). These regulatory proteins contain a Regulator of G Protein Signalling (RGS) domain and as such, are called RGS proteins (Woodard *et al.* 2015, Kehrl *et al.* 1998, Kach *et al.* 2012). They function by binding to active GTP-bound Gα subunits and accelerating hydrolysis activity by stabilizing the GTP to GDP transition state (Figure 1-6, Lin *et al.* 2014). RGS proteins may also exhibit other functional roles apart from their established effects on G protein activation, such as scaffolding or mediation of other effectors (Sato *et al.* 2006). Aside from RGS proteins, there are other effectors that exhibit GAP activity, specifically PLCβ, $G_{12/13}$-activated RhoGEFs but also some GRKs, which contain an RGS-like domain that may have limited activity (Ross *et al.* 2000, Siderovski *et al.* 2005).

Furthermore, the nucleotide-binding ability of Gα proteins can be influenced by non-receptor GEFs, which function by directly promoting the dissociation of GDP and consequently the binding of GTP. These include but are not limited to Activator of G Protein Signalling 1 (AGS1), Ric-8 proteins, β-APP, GAP-43 and PBP/RKIP, a Raf kinase inhibitor protein (Sato *et*

al. 2006, Zhao *et al.* 2013). Lastly, GDI proteins, which have only been discovered in the last two decades, influence nucleotide binding by reducing the exchange of GDP for GTP at Gα proteins both in the presence or absence of receptor activation (Cismowski, 2006). Additionally, these proteins have also been shown to modulate signal transmission by serving as alternative binding partners for Gα and Gβγ subunits. Examples include Nucleobindin 1 and Group II Activators of G Protein Signalling, such as GPSM3 (Kapoor *et al.* 2010, Cismowski, 2006). Additional GPCR regulation by these accessory proteins will be discussed in section 1.3.

The presence of certain toxins is known to disrupt GPCR signalling. One tool widely used in experimental research is pertussis toxin, a protein-based exotoxin initially discovered in *Bordetella pertussis* bacteria. Pertussis toxin is the main cause of the respiratory tract symptoms associated with pertussis infection such as whooping cough. The exotoxin is comprised of a six-protein complex: subunit A, which conveys enzymatic activity and five B subunits, which are collectively responsible for receptor-binding and initiating pathway for subunit A entry into the cell cytosol (Iwaki *et al.* 2015). The subunits are released from the bacteria in their inactive state and become functional after reformation of the active A-B complex within the cytosol (Plaut *et al.* 2016). This complex ADP-ribosylates $G\alpha_i$ proteins and thus, prevents interaction and signal transmission between the GPCR and heterotrimeric G proteins (Carbonetti *et al.* 2015, Chishiki *et al.* 2017). Therefore, pertussis-treated $G\alpha_i$ proteins remain in their inactive GDP-bound form and lose their ability to initiate downstream signalling.

Another toxin that works in a similar manner is cholera toxin (CTX), a protein complex produced and secreted by *Vibrio cholerae* bacterium. CTX is the culprit responsible for causing uncontrollable watery diarrhea in patients with cholera infection. The oligomeric toxin is

composed of six protein subunits, denoted as AB_5: one A subunit, with enzymatic activity, and five B subunits, with activities responsible for receptor binding. The five B subunit complex binds to target cells via GM1 gangliosides, a glycosphingolipid, which causes endocytosis of the entire toxin complex (Magnani *et al.* 1980). Endocytosis promotes a series of steps, which ultimately result in the release of subunit A from the oligomeric toxin by the reduction of disulfide bonds. The free subunit then catalyzes the ADP-ribosylation of the $G\alpha_s$ subunit, rendering it incapable of GTP hydrolysis and thus, maintaining it a constitutively active state (Cassel *et al.* 1977, Gill *et al.* 1978). Therefore, both PTX and CTX lead to increased cAMP production, albeit via different mechanisms.

In contrast to cholera toxin, which promotes G protein signalling via $G\alpha_s$, the mastoparan toxin, derived from wasp venom interferes with G protein activity by stimulating GTPase activity of G protein subunits, thereby reducing their lifespan (Higashijima *et al.* 1988). By the same token, mastoparans promotes GTP binding to the $G\alpha$ subunit by facilitating its dissociation of GDP (Weingarten *et al.* 1990). This mechanism of action by which this peptide toxin works follows from the fact that resembles an activated GPCR under certain conditions (*i.e.* within a phospholipid environment) (Jones *et al.* 2006).

In summary, G protein signalling can be modified through a myriad of different mechanisms. G protein activity can be altered through direct interactions with GAPs, GEFs and GDIs, which could accelerate or promote the inhibition of G protein activation. These binding interactions can alter proportions of free $G\alpha$ and $G\beta\gamma$ subunits, which in turn, could conceivably affect GRK activity by altering recruitment to the plasma membrane. Additionally, modulating the interaction between $G\beta\gamma$ and β-arrestins could influence β-arrestin-mediated signalling

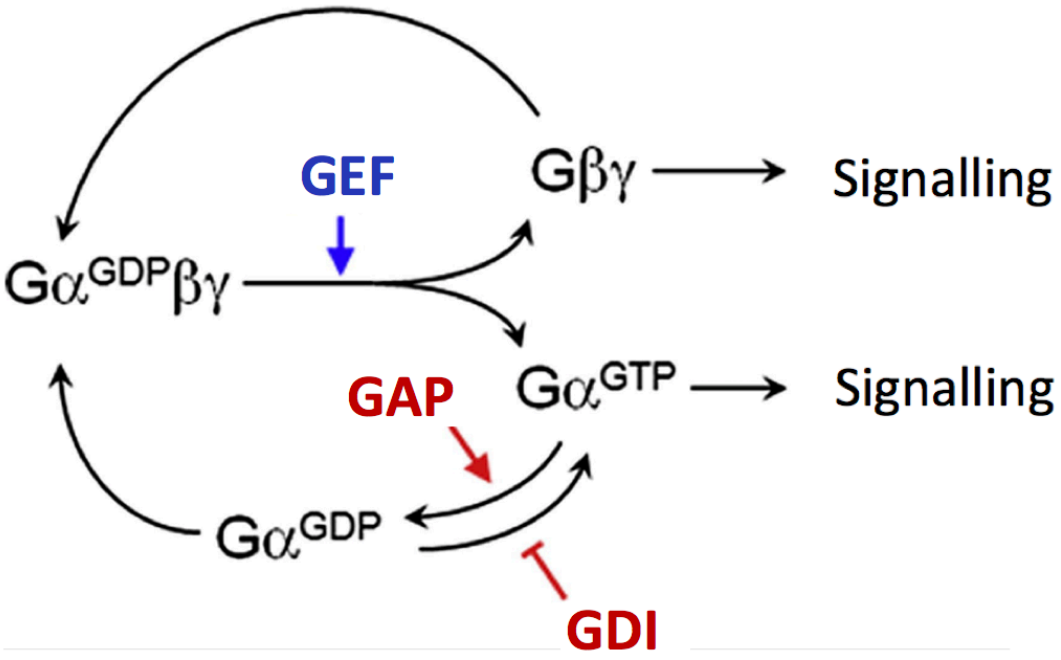

Figure 1-6. G protein regulation by accessory proteins. Guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs) and GTPase accelerating proteins (GAPs) modulate G protein signalling via nucleotides. GEFs and GDIs, specifically Group II AGS proteins, alter the rate of nucleotide exchange, whereas GAPs accelerate the rate of GTP hydrolysis.

Adapted from Lin *et al.* 2014

(Pitcher *et al.* 1992, Pitcher *et al.* 1998, Seitz *et al.* 2014). Moreover, G protein activation may also be influenced by biotoxins, such pertussis, cholera and mastoparans.

1.3 ACTIVATORS OF G PROTEIN SIGNALLING

1.3.1 *DISCOVERY OF AGS PROTEINS*

Several observations characterizing activation and inactivation states of G proteins have led to the finding that G protein signalling can occur independently of receptor activation. This implied that there could be third-party proteins mediating this effect. Various methods, such as protein interaction screens and biochemical assays, have been used to classify these intracellular regulators of G protein activity; however, out of the methods tested, a yeast-based screening assay designed within *Saccharomyces cerevisiae* was the most successful in isolating these signal regulators. This high throughput-screening assay developed by Lanier and colleagues took advantage of the endogenous GPCR-based pheromone mating pathway within *S. cerevisiae* yeast cells, which is canonically driven by Gβγ, subsequent to receptor activation (Cismowski, 2006). This pathway was modified by deleting the endogenous yeast GPCR in lieu for a pheromone-responsive reporter (Cismowski *et al.* 1999, Klein *et al.* 1998). To account for the fact that mammalian proteins probably interact better with mammalian rather than yeast G proteins, yeast Gα (*e.g.* $G\alpha_s$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{16}$) proteins were replaced with either human or rat counterparts, which proficiently couple to yeast Gβγ. Then, a variety of mammalian cDNA libraries were screened and selected based on their ability to activate this reporter via a simple growth assay. Proteins found to have the ability to activate this pheromone-responsive pathway were termed Activators of G protein Signalling (AGS) (Cismowski *et al.* 1999, Cismowski *et al.* 2006).

1.3.2 *CLASSIFICATION OF AGS PROTEINS*

Three groups of AGS proteins have been identified and divided according to their postulated mechanisms of action (Table 1.2) (Blumer *et al.* 2003, Blumer *et al.* 2005, Cismowski, 2006). Group I contains only a single member (AGS1, aka DexRas), which has been shown to exhibit GEF activity, similar to that of a GPCR, by increasing GTP binding to free $G\alpha_{i2}$ (Cismowski *et al.* 1999, Cismowski *et al.* 2000). Group II proteins are characterized as guanine nucleotide dissociation inhibitors (GDI) by the presence of G Protein Regulatory (GPR) or GPSM domains. These proteins influence nucleotide exchange at the level of the G protein. Lastly, members of Group III modulate the $G\alpha\beta\gamma$ complex by directly binding to $G\beta\gamma$.

1.3.3 *GUANINE NUCLEOTIDE DISSOCIATION INHIBITORS*

The GDIs that make up AGS group II are GPSM-containing proteins that can alter the lifetime of G protein activity. They contain one or more highly conserved 20-25 amino acid GPSM repeats that can modulate the heterotrimeric G protein complex via their interactions with GDP-bound $G\alpha_i$ proteins. Even though there is a general consensus regarding the biochemical nature of this repeat, there is no unanimity on its nomenclature (Zhao *et al.* 2013). The first protein ever discovered to contain this repeat was an RGS12 homologue called Loco, originally discovered in *Drosophila melanogaster* (Granderath *et al.* 1999). RGS12 contained a region that directly interacted with $G\alpha_i$ apart from its RGS domain, which led to classification of this region as $G\alpha_{i/o}$-Loco motif or for simplicity, GoLoco (Siderovski *et al.* 1999). In addition, the function of these domains as receptor-independent activators of the pheromone pathway in yeast led to the name G protein regulatory (GPR) domains (Takesono *et al.* 1999, Cismowski *et al.* 1999). While

GPR domain is sufficiently informative, it is also widely used in naming orphan GPCRs. Furthermore, this repeat was found to be shared among LGN proteins, which contain Leu-Gly-Asn-enriched motifs, and thus the name LGN repeat has also been used (Mochizuki *et al.* 1996, Ponting *et al.* 1999). The term GPSM is used by the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC) to denote multiple genes that encode proteins within this conserved GDI domain, specifically GPSM1 (AGS3), GPSM2 (AGS5, LGN) GPSM3 (AGS4, G18) and GPSM4 (Purkinge Cell Protein 2, L7) (Bruford *et al.* 2018). Thus, based on the HGNC dictates, we prefer to use the term *GPSM domain* to describe these conserved motifs (Zhao *et al.* 2013)

This selective binding promotes stability of the GDP-bound conformation and at the same time, impedes the exchange of GDP for GTP (Figure 1-7). In addition, GDIs can compete with the Gβγ subunit for the $G\alpha_i$ subunit (Dirac-Svejstrup *et al.* 1997). For that reason, the Gβγ subunit can be considered as a GDI by stabilizing GDP-Gα and preventing the activation of free Gα proteins (Neer *et al.* 1995, Tang *et al.* 2006).

The GPSM motif binds to the GDP-bound form of $G\alpha_{i/o}$ with a higher affinity when compared to GTP-bound (Kimple *et al.* 2002). As a result, they can promote G protein heterotrimer dissociation from the initial preformed complex independently of receptor activation (Blumer *et al.* 2005, Bernard *et al.* 2001, Natochin *et al.* 2001). Furthermore, this GDI-binding induces a conformational change in the switch region of Gα subunit, thereby preventing re-association of Gβγ to the Gα subunit (Natochin *et al.* 2001, Bernard *et al.* 2001, Siderovski *et al.* 2005). This phenomenon has been elucidated with a GPSM-containing peptide derived from AGS3. It has been shown that this peptide prevents Gα re-association to the Gβγ

dimer with 10 times greater potency than a Gβγ-specific peptide, called Gβγ hot spot-binding peptide (SIGK) (Ghosh *et al.* 2003). Moreover, observations with this peptide were also able to support the idea that GDIs can promote G protein subunit dissociation, as it increased the rate of Gβγ dissociation from Gα by 13-fold when compared to the intrinsic rate of heterotrimer dissociation (Ghosh *et al.* 2003).

1.3.4 *MOLECULAR BASIS FOR FUNCTIONAL ACTIVITY OF GDI*

The binding interaction between the GPSM motif of RGS14 and $G\alpha_{i1}$ has been structurally resolved using crystallography. This structural information reinforces the importance of the highly conserved Asp/Glu-Gln-Arg triad in GPSM motifs and the switch II region of $G\alpha_i$ proteins (Kimple *et al.* 2002a). The N-terminal domain of the GPSM motif forms an α helix which positions itself between the α3 helix and switch II regions of $G\alpha_i$. This binding displaces the switch II region of the $G\alpha_i$ protein away from the α3 helix, thereby deforming of the Gβγ-binding site. Moreover, the triad region within the GPSM domain is part of an arginine finger motif, which directly interacts with the nucleotide binding pocket within $G\alpha_i$ via α and β phosphates along the bound GDP (Peterson *et al.* 2000, Kimple *et al.* 2002a). An arginine to phenylalanine substitution mutation within this triad leads to a complete loss of function. Interestingly, an arginine substitution to a less bulky hydrophobic residue allows for partial GDI function (Peterson *et al.* 2000, Kimple *et al.* 2002, Takesomo *et al.* 1999, Bernard *et al.* 2001). This suggests that the triad is a significant, but not a necessarily essential determinant of GDI activity (Willard *et al.* 2004). Furthermore, the glutamine residue preceding the arginine residue also contributes to activity by facilitating important interactions with the $G\alpha_i$ subunit for proper

Table 1.2. Classes of AGS proteins and their associated properties

AGS Families	Structure	Gα-subtype selectivity	Alternative Names
Group I (GEF)			
AGS1	Ras-related	$G\alpha_{i2}$, $G\alpha_{i3}$	Dexras1, RASD1
Group II (GDI)			
AGS3	Four GPSM motifs	$G\alpha_{i2}$, $G\alpha_{i3}$	GPSM1
AGS4	Three GPSM motifs	$G\alpha_{i2}$, $G\alpha_{i3}$	GPSM3
AGS5	Three GPSM motifs	$G\alpha_{i3}$	GPSM2, LGN
AGS6	One GPSM motif	$G\alpha_{i3}$	RGS12
Group III			
AGS2	Binds βγ	$G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{s}$, $G\alpha_{i16}$	Tctex-1, DYNLT1
AGS7	Binds βγ	$G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{s}$, $G\alpha_{i16}$	TRIP13
AGS8	Binds βγ	$G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{s}$, $G\alpha_{i16}$	KIAA1866, FNDC1

Adapted from Cismowski *et al.* 2006

GPSM-nucleotide binding activity. Mutating this residue leads to loss of GDI activity. Finally, the acidic residues within the triad are necessary to structurally support the glutamine residue (Willard *et al.* 2004).

The binding of the GPSM domain to Gα subunits additionally displaces Arg^{178}, which is located within the switch I region of $G\alpha_I$. This binding allows for direct interaction with the phosphate group, resulting in a newly formed contact with the GDP ribose group. This contact is thought to underlie the molecular basis of GPSM GDI activity (Kimple *et al.* 2002a). The helical domains of Gα subunits and core GPSM motifs collectively contribute to the selectivity among different G proteins and GPSM-containing proteins.

1.3.5 *REGULATION OF GDI ACTIVITY*

A large proportion of GPSM-motif containing proteins contain serine and threonine residues within the N-terminal region. These residues can be subject to phosphorylation by protein kinases, which in turn can alter protein functional activity. Phosphorylation of RGS14 at residue Thr-494 by PKA significantly increases its GDI activity (Hollinger *et al.* 2003). On the other hand, phosphorylation of GPSM2 (Thr-450) by liver kinase β1 (LKB1) significantly decreases its ability to bind with $G\alpha_i$ proteins (Blumer *et al.* 2003). This suggests that phosphorylation is one post-translational modification that is able to regulate GDI activity and the primary outcomes of such changes strongly depends on the type of GPSM protein and the cellular environment (Blumer *et al.* 2007). Furthermore, whether or not such phosphorylation directly affects activity or disrupts the interaction between $G\alpha_i$ proteins and the GPSM motif is poorly understood (Willard *et al.* 2004).

Besides phosphorylation, the interaction between GPSM-containing proteins and $G\alpha_i$ can be influenced by GPCR activation and by the $G\beta\gamma$ dimer. Activation of $G\alpha_i$-coupled receptors, α_2-adrenergic and μ-opioid, led to a reduction in the measured BRET signal between GPSM1 and $G\alpha_i$. This decrease was reversed by overexpression of RGS4, which rapidly hydrolyzes GTP to GDP at the $G\alpha$ subunit, implying that the GPSM1 binds more favourably to the GDP-bound form of $G\alpha_i$ rather than the GTP-bound form. This strongly suggests that nucleotide exchange and hydrolysis could potentially mediate this effect (Oner *et al.* 2010a). Correspondingly, coupling behavior between GPSM3 and $G\alpha_{i/o}$ was reduced shortly after α_2 adrenergic receptor activation, which could be related to which nucleotide is bound to the G protein; however, the mechanism and significance of this effect is poorly understood (Oner *et al.* 2010a, Oner *et al.* 2010b).

Biochemically, GDI proteins reduce receptor-induced nucleotide exchange, as indicated by decreases in steady-state GTPase assays and also pre-steady state GTPγS binding assays (Natochin *et al.* 2000, Kerov *et al.* 2005, Zhao *et al.* 2010). Depending on the cellular context, receptor type and GPSM protein being studied, the GDI activity may also be weakened by slow GTP hydrolysis (rate limiting step in G protein activation) (Hepler *et al.* 2005, Zhao *et al.* 2013).

1.4 G PROTEIN SIGNALLING MODULATOR-3

GPSM3, also known as G18 and AGS4, is a small 160-amino acid long protein. GPSM3 is structurally comprised of a short proline-rich N-terminal domain and three tandem GPSM motifs. GPSM3 was the fourth protein discovered in the early yeast-based screening assays for

the detection of GPSM domain-containing proteins (Cismowski *et al.* 2006, Zhao *et al.* 2010). In the yeast-based screen for AGS proteins, cDNA libraries from human heart and prostate leiomyosarcoma (GPSM3-expressing tissues) were used in the sets of experiments from which GPSM3 was identified (Cismowski *et al.* 1999). The GPSM3 gene is encoded within the major histocompatibility complex class III region of human chromosome 6 (Kimple *et al.* 2004, Cao *et al.* 2004). Its expression within the body occurs mainly in immune cells involved in inflammation, but has also been found elsewhere, such as vascular smooth muscle cells, placenta, heart, liver, lung and within podocytes of the kidney (Zhao *et al.* 2015, Giguere *et al.* 2013, Cao et al. 2004).

1.4.1 *MODULATION OF GPCR SIGNALLING*

GPSM3 is a GDI that functions to decrease the rate nucleotide exchange at the G protein. It binds to GDP-bound forms of $G\alpha_i$ via its GPSM motifs. In this manner, GPSM3 bears the potential to inhibit $G\alpha_i$ activation by slowing down the rate of GDP dissociation from the $G\alpha_i$ subunit. At the same time, this interaction impedes association between $G\alpha_i$ to its $G\beta\gamma$ counterpart by occluding the $G\beta\gamma$ binding site. This in turn, could promote $G\beta\gamma$-mediated signalling within the cell (Cismowski *et al.* 2006, Zhao *et al.* 2013, Siderovski *et al.* 2005).

The binding interactions between GPSM3 and $G\alpha_i$ proteins could potentially serve multiple functions. Evidence derived from a series of yeast-two-hybrid assays suggest that GPSM3 can bind to all conventional $G\alpha_i$ proteins: $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ (Giguere *et al.* 2012). Similar studies suggest that full length GPSM3 can bind to fluoroaluminate-activated forms of $G\alpha_{i1}$ and $G\alpha_o$ (Zhao *et al.* 2010), in addition to its established binding to inactive GDP-bound form of $G\alpha_{i1}$.

The binding of the GPSM motifs to the Gα proteins reduces the rate of nucleotide exchange from $G\alpha_i$ proteins (Kimple *et al.* 2004, Zhao *et al.* 2010), whereas the N-terminal domain serves as a GEF for $G\alpha_{i1}$ (Zhao *et al.* 2010). There is also evidence that the N-terminal has weak GDI activity on Ga_o but with very low potency. Furthermore, the GEF activity exhibited by the N-terminus does not require functional GPSM motifs, at least in the case of $G\alpha_o$ (Zhao *et al.* 2010). Studies examining local energy transfer between proteins have proposed that GPSM/$G\alpha_i$ binding interaction can contribute to GPCR signalling by positioning Gα subunits to proximal regions of receptors for activation (Oner *et al.* 2010b).

In light of research performed in our lab, there is evidence suggesting that GPSM3 could have additional biochemical activity apart from its GDI function. A series of GTPγS binding assays have been conducted to determine the rate of nucleotide exchange among G proteins (Willard *et al.* 2008, Zhao *et al.* 2010). A previous study from our lab showed that the N-terminal region of GPSM3 exhibits GEF activity for $G\alpha_{i1}$ (Zhao *et al.* 2010). Therefore, GPSM3 would appear to have the capacity to simultaneously inhibit and activate $G\alpha_{i/o}$ signalling while indirectly promoting Gβγ signalling (Blumer *et al.* 2012, Guiguere *et al.* 2012).

Despite the well-established binding interactions between GPSM3 and Gα proteins, there is still some uncertainty about whether or not GPSM3 can also bind to Gβγ. Co-immunoprecipitation studies published by Siderovski and his team suggest that GPSM3 can directly bind to all four isoforms of Gβ (Gβ1, Gβ2, Gβ3, Gβ4) via an interaction independent of the established GPSM/$G\alpha_i$ binding (Giguere *et al.* 2012). Interestingly, GPSM3 was not found to bind to the Gγ subunit. Moreover, there is evidence that ectopic GPSM3 expression within THP-1 cells negatively influences GPCR signal transduction via PLCβ. In these experiments,

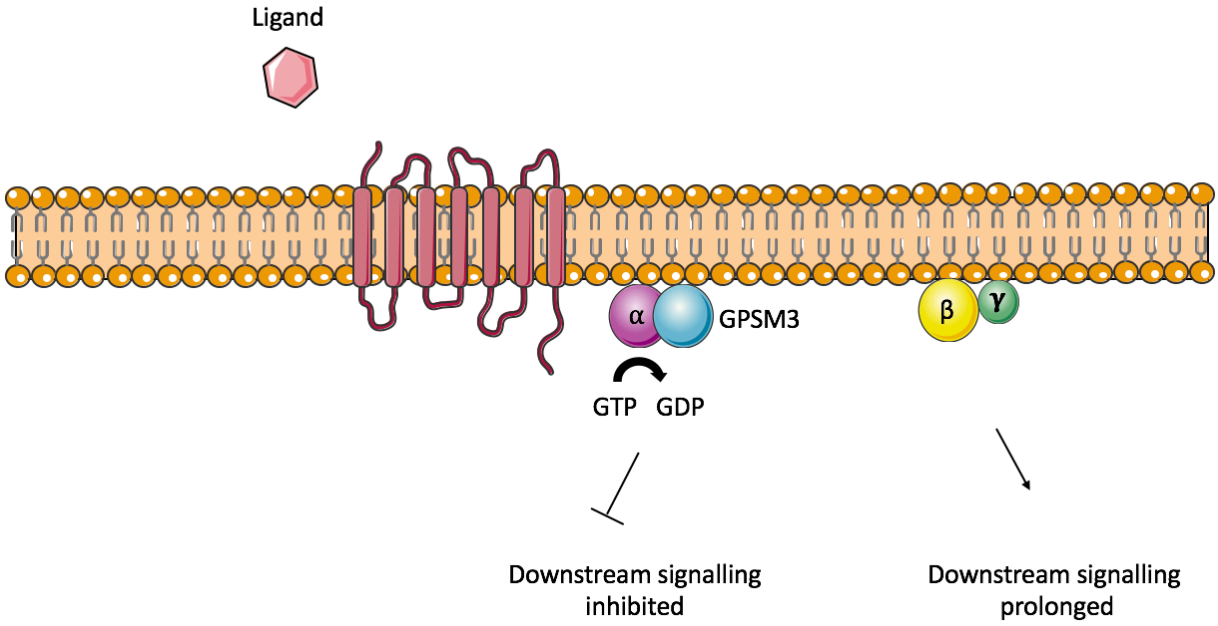

Figure 1-7. GPSM3 binds to and inhibits $G\alpha_I$-dependent signalling while prolonging $G\beta\gamma$-dependent signalling. GPSM3 binds to the GDP-bound form of $G\alpha_I$ proteins via its GPSM motifs and prevents nucleotide exchange from GDP to GTP. Furthermore, the complex formed by GPSM3 and $G\alpha_I$ prevents re-association of the $G\beta\gamma$ dimer to the $G\alpha_I$ subunit, thereby prolonging $G\beta\gamma$-dependent signalling.

GPSM3 has been reported to decrease IP_3 production indirectly by binding to $G\beta\gamma$ and

preventing its activation of PLCβ. In addition, this effect was reversed by overexpressing free $G\beta_1\gamma_2$ subunits and using a version of GPSM3 bearing a loss of function mutation within the leucine-rich region that appears to be responsible for Gβ-binding (Giguere *et al.* 2013). However, this basic finding has not been reported by other labs and we were unable to reproduce it within our lab as well (Wallace, D, 2017, MSc thesis, University of Western Ontario). Furthermore, GPSM3 plays a role in downstream signalling via GPCR/Gβγ-mediated activation of PI3K within immune cells (Easton *et al.* 2007, Barberis *et al.* 2008). Studies indicate that low GPSM3 expression levels have been linked to diminished survival in monocytic THP-1 cells (Giguere *et al.* 2013).

GPSM3 has been shown to interact with a variety of other proteins, including receptors, separate from the heterotrimeric G protein complex. There is evidence that some RGS and GPSM-containing proteins can directly and indirectly bind to GPCRs (Abramow-Newerly *et al.* 2006, Oner *et al.* 2010a). A study by Zhao *et al.* 2015 has identified RGS5 as a novel binding partner for GPSM3, and that both the GPSM domain-containing portions of GPSM3 are crucial for its ability to facilitate this interaction. In contrast, there was no evidence for interactions between GPSM3 and RGS2, RGS4 or RGS16 in the same study. An additional study by Giguere *et al.* 2012 carried out a series of yeast two-hybrid screening assays using a cDNA library from human leukocytes. In these experiments, GPSM3 was shown to interact with several isoforms of 14-3-3, a conserved eukaryotic protein that regulates function of diverse signalling molecules, such as kinases, phosphatases and RGS proteins (Fu *et al.* 2000).

As previously mentioned, GPSM3 consists of three GPSM domains; however, conflicting data exists regarding whether or not all three domains are functionally active. Studies by Willard *et al.* 2004 and Kimple *et al.* 2004 attributed biochemical activity of GPSM3 specifically to the first and third GPSM motifs through a series of co-immunoprecipitation pull down assays with different $G\alpha_i$ isoforms. They have ruled out the activity of the second motif due to discrepancy within the critical Asp/Glu-Gln-Arg triad that is normally conserved among all GPSM motifs (Giguere *et al.* 2011). On the other hand, a study examining the interactions of GPSM3 with $G\alpha_i$ using BRET has reported that all three GPSM motifs exhibit GDI activity (Oner *et al.* 2010a).

Despite the established binding interactions of GPSM3, it is still unclear how this protein modulates GPCR signalling pathways. Effects on signalling have been theoretically proposed; however, little functional data exists on GPSM3 activity. One way to examine how the interaction between GPSM3 and $G\alpha_i$ proteins influences GPCR signalling is to analyze changes in adenylyl cyclase activity via direct measurements of cAMP production. Moreover, there is virtually no knowledge on whether or not GPSM3 can impact the processes of receptor desensitization or G protein-independent signalling. Given that GPSM3 can sustain the inactive conformation of $G\alpha_i$ and also simultaneously activate $G\beta\gamma$, it is possible that this interaction would affect signal transduction pathways, perhaps by altering $G\beta\gamma$ or β-arrestin signalling.

1.4.2 *CELLULAR FUNCTIONS OF GPSM3*

Little is known regarding how GPSM3 affects GPCR signalling. To date, we know that GPSM3 functions as a GDI by selectively interacting with GDP-$G\alpha_i$ via its GPSM domains and

as a GEF by selectively interacting with $G\alpha_o$ via its N-terminus, respectively, *in vitro* (Giguere *et al.* 2013, Zhao *et al.* 2010). In addition, there is conflicting evidence regarding whether GPSM3 overexpression affects the activity of PLCβ via a Gβγ-dependent mechanism. In a series of Bioluminescence Resonance Energy Transfer (BRET) experiments conducted by Oner *et al.* 2010a, GPSM3 was tagged with *Renilla luciferase* (RLuc), while $G\alpha_{i1}$ and α_2-adrenergic receptor were tagged with yellow fluorescent protein (YFP) and venus, respectively, to measure interactions between each other. The study reported that the BRET signal between GPSM3 and $G\alpha_{i1}$ after receptor activation with the agonist UK 14,304 was reduced, and subsequently blocked with pertussis toxin. In addition, the BRET signal between GPSM3 and α_2-adrenergic receptor was also reduced by agonist. These two observations collectively suggest that the complex formed by GPSM3 and $G\alpha_i$ is likely to be receptor-proximal, with an ability to directly couple to the GPCR. Furthermore, the GPSM3-$G\alpha_i$ complex could also potentially serve as a substrate for agonist-induced activation of $G\alpha_i$, with GPSM3 substituting for Gβγ in the heterotrimeric G protein complex (Oner *et al.* 2010b).

It is widely recognized that GPSM3 is a cytosolic protein that can localize to the plasma membrane (often co-localized with $G\alpha_i$ proteins) and less often, to the nucleus (Cao *et al.* 2004, Giguere *et al.* 2012, Zhao *et al.* 2015). In addition to its established binding interactions with $G\alpha_i$ family proteins, the GPSM3-$G\alpha_i$ complex may directly couple to GPCRs, specifically the α_2-adrenergic and μ-opioid receptors (Oner *et al.* 2010b). Other evidence suggests that GPSM3 interacts with RGS5 and regulates its GAP activity (Zhao *et al.* 2015). Furthermore, complex interactions with NOD-like receptor family, heat shock A8 protein and pyrin domain containing protein 3 have been observed (Giguere *et al.* 2014). Interestingly, GPSM3 interactions with 14-3-

3 proteins appears to stabilize the structure of GPSM3, potentially by influencing various phosphorylation sites along the protein (Giguere *et al.* 2012, Bian *et al.* 2014).

1.4.3 *PHYSIOLOGICAL FUNCTIONS OF GPSM3*

GPSM3 has been implicated in multiple human diseases, specifically inflammation and autoimmune disorders, such as rheumatoid arthritis, dermatitis, systemic lupus, ulcerative colitis and ankylosing spondylitis (Barcellos *et al.* 2009, Pathan *et al.* 2009, Giguere *et al.* 2013). Additionally, GPSM3 expression has been linked to other diseases, specifically prostate leiomyosarcoma and polycystic kidney disease (Gall *et al.* 2016, Lenarczyk *et al.* 2015). Furthermore, there is recent evidence for its role in the formation of mitotic spindles during cell division (Wallace, D, 2017, MSc thesis, University of Western Ontario). GPSM3 expression is primarily restricted to leukocytes, myeloid-derived cells and lymphoid organs, such as the spleen, where expression levels appear to be somewhat dependent on external stimuli (Gall *et al.* 2016). Its role within these cells has been studied by several labs and their work has collectively shown the link between GPSM3 to autoimmune and inflammatory diseases (Giguere *et al.* 2013, Giguere *et al.* 2014, Billard *et al.* 2014, Gall *et al.* 2016, Robichaux *et al.* 2017).

Recent research on GPSM3 has focused on its underlying function in immune cells. Monocyte and macrophage differentiation is important in inflammatory responses and recent evidence demonstrates that expression of GPSM3 is tightly regulated during these steps. *In vitro* and *in vivo* studies have shown that GPSM3 deficiency significantly decreases monocyte number, survival and slows down migration to specific chemokines (Giguere *et al.* 2013, Sunderkotter *et al.* 2004). This strongly suggests that GPSM3 regulates GPCR signalling at some

level, although the underlying mechanisms have not been investigated. This phenomenon has been shown using $Ly6C^{high}CD11b^{+}$ monocytes, a subset population of inflammatory cells that rapidly mobilize to sites of infection. Additionally, $GPSM^{-/-}$ mice have been shown to exhibit a higher resistance to a monocyte-driven model of acute inflammatory arthritis when compared with wildtype control mice. Collectively, these observations suggest that GPSM3 could be involved in promoting differentiation and survival of specific myeloid-derived cells, which could in turn promote inflammatory processes.

Despite its well-established expression within monocytes, GPSM3 is also present in prostatic cancer cells. A study by Lapan *et al.* has shown that when prostate cancer cells are grown in an environment mimicking tumorigenesis or angiogenesis, GPSM3 levels increase two-fold. Although the mechanism by which this occurs is unclear, this suggests that GPSM3 could not only influence migratory potential of cancer cells but also alter cellular responses to different survival or angiogenic stimuli released by cancer-causing cells.

In recent years, genome-wide association studies have uncovered several genetic polymorphisms within the GPSM3 gene that suggest it may be linked to prevalence of certain diseases. GPSM3 polymorphisms rs204989 and rs204991 have been characterized to decrease the incidence of certain autoimmune diseases like rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and systemic lupus erythematosus (Wellcome Trust Case Control Consortium *et al.* 2007, Sirota *et al.* 2009, Corona *et al.* 2010, Gall *et al.* 2016). A recent study has reported allele frequencies for GPSM3 polymorphisms rs204989 and rs204991 of 23% within a cohort of patients studied with rheumatoid arthritis while only 18% in disease-free controls (Gall *et al.* 2016). Conversely, these polymorphisms have also been shown to increase prevalence of type I diabetes, autoimmune thyroid disease and chronic inflammation, often seen in childhood obesity

and atopic dermatitis (Comuzzie *et al.* 2012, Chang *et al.* 2012).

Current treatment for inflammatory diseases focuses on neutralizing pro-inflammatory molecules by decreasing chemokine production and monocyte migration. Additional research must be centered on targeting these inflammatory pathways and preventing their exacerbation. One possible way to study this is by examining similarities between these cells types and determining which factors promote their actions. Given that GPSM3 expression occurs mainly within hematopoietic tissues, it makes sense to posit that its role within these tissues is important. Studies show that viability and chemotaxis appear to be decreased in THP-1 monocyte populations devoid of GPSM3 in the presence of chemokine, CCL3, CX3CL1 and chimerin (Giguere *et al.* 2013). Furthermore, GPSM3-deficient mice appear to be protected from acute, collagen antibody-induced arthritis (CIA) and exhibit less synovial damage, bone erosion and inflammation when compared with their wildtype counterparts. Additionally, when wildtype mice were treated with dexamethasone, they appeared to have reduced levels of GPSM3 (Schmidt *et al.* 2012). Interestingly, it has been reported that agonists, specifically Interleukin-6 and Interleukin-1β, and chemokine receptor expression are both reduced in GPSM3 knockout mouse models (Giguere *et al.* 2013). This in turn has been shown to correlate to severity of autoimmune diseases by directly reducing monocyte chemotaxis towards specific chemokines (CCL2, chimerin, CX3CL1). Therefore, we need a better understanding of GPSM3 signalling to determine whether this protein could serve as a therapeutic target for patients with autoimmune disorders.

1.5 RATIONALE

Canonically, GPSM3 should be able to modulate G protein activity by binding to and inhibiting $G\alpha_i$ signalling, while concurrently promoting $G\beta\gamma$ signalling. There is evidence that the $G\beta\gamma$ dimer can interact with multiple effector proteins as well as GRK and β-arrestin proteins, and the latter two play key roles in β-arrestin-mediated receptor internalization. Therefore, prolonged $G\beta\gamma$ signalling via GPSM3 could influence receptor desensitization by fostering interactions with GRKs and β-arrestins.

1.5.1 *OBJECTIVES*

Objective 1: To determine whether GPSM3 alters β-arrestin recruitment to $G\alpha_i$-, $G\alpha s$- or $G\alpha_{q/11}$-coupled receptors after stimulation with receptor-specific agonists

Objective 2: To ascertain which protein domains of GPSM3 are important for its effects on GPCR signalling

Objective 3: To analyze whether GPSM3 alters the ability of G_i-coupled receptors to inhibit adenylyl cyclase activity

1.5.2 *HYPOTHESIS*

I hypothesize that G Protein Signalling Modulator-3 will decrease $G\alpha_i$-dependent signalling while promoting $G\beta\gamma$-dependent signalling in response to activation of G_i-coupled GPCRs, but not G_s- or G_q-coupled GPCRs.

The proposed studies aim to elucidate the role of GPSM3 on GPCR signalling in human cells. The study of how GPSM3 can influence receptor desensitization will help us further understand basic aspects of β-arrestin-mediated signalling and possibly discover another role for GPSM-containing proteins. It will also give us a better idea of how GPSM3 can regulate signalling and possibly uncover novel targeting sites for future therapeutic drugs.

Chapter 2

2 MATERIALS AND METHODS

2.1 *MATERIALS AND SOLUTIONS*

Dulbecco's Modified Eagle Medium (DMEM), heat-inactivated fetal bovine serum (FBS), antibiotic solution (10,000 U/mL penicillin, 10,000 μg/mL streptomycin), trypsin solution, minimum essential medium (MEM; without bicarbonate, and with or without phenol red), and Dulbecco's phosphate-buffered saline (DPBS) were obtained from Thermo Fisher Scientific (Waltham, MA). X-tremeGENE9 Reagent was obtained from Roche Diagnostics (Laval, QC, Canada). GloSensor™ cAMP Reagent was obtained from Promega (Madison, WI). Bovine albumin (BSA) was obtained from MP Biomedicals (Solon, OH). 3-isobutyl-1 methylxanthine (IBMX) and UK 14,304 were obtained from Sigma-Aldrich (St. Louis, MO). D-luciferin sodium salt was obtained from Gold Biotechnology (St. Louis, MO). 2-Chloro-N(6)-cyclopentyladenosine (CCPA) was purchased Sigma-Aldrich (St. Louis, MO. 7-Deacetyl-7-[O-(N-methylpiperazino)-γ-butyryl]-Forskolin Dihydrochloride (DMB-Forskolin) was purchased from Santa Cruz Biotechnology (Dallas, TX). [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO) was purchased from Tocris BioScience (Bristol, UK). Rat PTH (1-34) was purchased from Bachem (Bubendorf, Switzerland). Peptides were dissolved and diluted in Dulbecco's phosphate-buffered saline (DPBS), supplemented with 0.1% Bovine Serum Albumin (BSA).

Table 2.1 Plasmid constructs used

Plasmid	Description
pGloSensor-22F cAMP	Luciferase-based cAMP biosensor (#)
CMV6-hGPSM3wt	Full length wildtype human GPSM3 (•)
CMV6-hGPSM3mGL	Full length human GPSM3 containing R to F mutations in the last amino acid of each GPSM motif (•)
CMV6-hGPSM3•δN•mGL	Human GPSM3 with an N-terminal truncation in addition to R to F mutations in the last amino acid of each GPSM motif (•)
CHRM1-linker20-PtGRC394 in pcDNA4 V5/His(B)	Full length wildtype rat muscarinic type 1 receptor tagged with a C-terminal luciferase fragment (*)
ADRB2-linker 20-PtGRC394 in pcDNA4 V5/His(B)	Full length wildtype human β_2-adrenergic receptor tagged with a C-terminal luciferase fragment (*)
pcDNA3.1(+)- (empty vector)	Backbone vector used as a control
ADRA2A-PtGRC394 in pcDNA4 V5/His(B)	Full length wildtype human α_{2A}-adrenergic receptor tagged with a C-terminal luciferase fragment (*)
hPTH1R-linker20-PtGRC394 Myc/His(B) in pcDNA3.1	Full length wildtype human parathyroid hormone type-1 receptor tagged with a C-terminal luciferase fragment (#)
OPRm1-linker 20-PtGRC394 in pcDNA4 V5/His(B)	Full length wildtype human μ-opioid receptor tagged with a C-terminal luciferase fragment (*)

PtGRN415-ARRB1 in pcDNA3.1 Myc/His(B)	Full length wildtype human β-arrestin isoform type-1 tagged with an N-terminal luciferase fragment (*)
pcDNA3-ratADCY3-QMP1	Full length wildtype rat adenylyl cyclase isoform III (♦)
pcDNA3-ratADCY4-QMP2	Full length wildtype rat adenylyl cyclase isoform IV (♦)
pcDNA3.1(+)-hGβ1-GNB00100000	Full length wildtype human G protein β subunit isoform type-1 (♦)
pcDNA3.1(+)-hGγ2	Full length wildtype human G protein γ subunit isoform type-2 (♦)
pcDNA3.1-PTHR1	Full length wildtype human parathyroid hormone type-1 receptor (#)

Plasmid sources:

• Dr. David P. Siderovski (The University of North Carolina, Chapel Hill NC, USA)

* Dr. Takeaki Ozawa (The University of Tokyo, Bunkyo, Tokyo, Japan)

Kim B *et al*. 2018

♦cDNA Resource Center (Bloomsberg University of Pennsylvania, Pennsylvania, USA)

2.2 *CELL CULTURE CONDITIONS*

All experiments were conducted using HEK-293H (human embryonic kidney) cell line from Thermo Fisher Scientific (Gibco® 293-H). Cells were subcultured twice weekly in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic (10,000 U/mL penicillin, 10,000 µg/mL streptomycin) solution and maintained at 37°C and 5% CO_2.

2.3 *TRANSFECTION CONDITIONS*

All transfections were conducted using X-tremeGENE 9 Reagent as per the manufacturer's protocol. Each DNA transfection complex was prepared by mixing 97 µl of DMEM, 3 µl X-tremeGENE 9 Reagent, and a combination of plasmid vectors (total sum of 1 µg). This complex was then incubated at room temperature for 20 mins. Next, cell suspensions were prepared by washing cells with PBS, lifting cells using trypsin and resuspension in fresh medium. The DNA transfection complex was then added directly to the suspension and thoroughly mixed before being plated into 96-well plates at a seeding density of 5.0×10^4 cells/well (1.5×10^5 cells/cm^2) as indicated for each experiment. Transfection efficiencies ranged between 70-80% in individual experiments.

2.4 *REAL-TIME cAMP MEASUREMENT*

HEK293-H cells from Thermo Fisher Scientific (Gibco® 293-H) were co-transfected with three plasmids – GloSensor™-22F cAMP biosensor plasmid, a plasmid vector encoding a receptor of interest and pCMV6-FLAG-hGPSM3 (full length human wildtype GPSM3) or

pcDNA3.1(+) (empty control vector) – and seeded on solid white 96-well plates at a density of 5.0×10^4 cells/well (1.5×10^5 cells/cm^2). Cells were then incubated at 37°C/5% CO_2 for 24 hours. Following incubation, medium was aspirated and replaced with fresh MEM, supplemented with 2 mM D-luciferin, 20 mM HEPES and 0.1% BSA (w/v) (pH = 7.20 ± 0.02; 300 ± 5 mOsmol/L).

G_i-COUPLED GPCR

Following a two-hour incubation with fresh MEM, supplemented with 2 mM D-luciferin, 20 mM HEPES and 0.1% BSA (w/v) (pH = 7.20 ± 0.02; 300 ± 5 mOsmol/L) at room temperature, cells were pre-treated with IBMX (500 μM) for 15 mins and receptor-specific agonist for 5 mins; cells co-transfected with adenosine-A1 receptor were stimulated with 2-Chloro-N(6) cyclopentyladenosine (CCPA) (10 pM – 10 μM), whereas cells co-transfected with α_{2A}-adrenergic and μ-opioid receptors were stimulated with UK 14,304 (10fM – 1nM) and [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO) (100 pM – 100 μM), respectively. Then, cells were stimulated with DMB-Forskolin (final concentration of 2 μM), at room temperature.

G_s-COUPLED GPCR

Following a two-hour incubation with fresh MEM, supplemented with 2 mM D-luciferin, 20 mM HEPES and 0.1% BSA (w/v) (pH = 7.20 ± 0.02; 300 ± 5 mOsmol/L) at room temperature, cells were pre-treated with IBMX (500 μM) for 20 mins and then stimulated with receptor-specific agonist, at room temperature; cells co-transfected with β_2-adrenergic receptor were stimulated with adrenaline/epinephrine (100 pM – 100 μM) and cells co-transfected with parathyroid hormone type-1 receptor were stimulated with PTH (1-34) (1 pM – 1 μM), respectively.

Luminescence was measured using an LMax^{TM}II^{384} for 45 mins at room temperature with an integration time of 1 second and interval of 1 minute.

2.5 *REAL-TIME β-ARRESTIN-1 RECEPTOR INTERACTION*

A real-time complementation-based luminescence assay was used to examine changes in β-arrestin recruitment to G_i, G_s and $G_{q/11}$-coupled receptors in the presence or absence of GPSM3, following stimulation with a selective agonist (Misawa *et al.* 2010). All plasmids were generously provided by Dr. Takeaki Ozawa (University of Tokyo, Japan), with the exception of the parathyroid hormone type 1-based construct, which was made in our lab by Dr. Alexey Pereverzev (Kim *et al.* 2018). Cells were co-transfected with β-arrestin-1 (modified at its N-terminus with amino acid residues of emerald click beetle luciferase) and receptor of interest (modified at its intracellular C-terminus by the addition of amino acid residues of emerald click beetle luciferase). Cells were co-transfected with both plasmids in the presence of GPSM3 or control vector. In response to receptor activation by a selective agonist, the N-terminal click beetle luciferase-β-arrestin-1 is recruited to the click beetle luciferase complement encoded on the C-terminus of the receptor of interest. When both halves of the chimeric proteins come in close proximity to each other, the complementary luciferase fragments reconstruct the functional luciferase enzyme.

HEK-293H cells were co-transfected with the following plasmids: β-arrestin-1, receptor of interest (α_{2A}-adrenergic, μ-opioid, β_2-adrenergic, parathyroid hormone type-1 or muscarinic type-1 receptors) and GPSM3 or control vector plasmids (1:1:2 molar ratio) in suspension and plated at a seeding density of 5 x 10^4 cells/well (1.5 x 10^5 cells/cm^2) on solid white bottom 96-

well plates (Corning). After a 24-hour incubation period at 37°C and 5% CO_2, old medium was aspirated and replaced with 90 µl of fresh phenol red-free MEM (supplemented with 3.2 mM D-luciferin, 20 mM HEPES, and 0.1% BSA; pH = 7.20 ± 0.02; 300 ± 5 mOsmol/L). Cells were then incubated for one hour at 37°C. Following incubation, cells were stimulated with agonist selective for receptor of interest at time 0; α_{2A}-adrenergic receptor was stimulated with UK 14,304, µ-opioid receptor was stimulated with DAMGO, β_2-adrenergic receptor was stimulated with adrenaline/epinephrine, parathyroid hormone type-1 receptor was stimulated with PTH (1-34) and muscarinic type-1 receptor was stimulated with carbachol. Luminescence was measured using LMax^{TM}II^{384} for 80 mins at 37°C with an integration time of 2 seconds and interval of 2 minutes.

2.6 *GPSM3 MUTANT CONSTRUCTS*

Flag-tagged human GPSM3 wildtype (full length GPSM3), GPSM3mGL containing an arginine to phenylalanine single point mutation in the last amino acid of each GPSM motif) and ΔN•GPSM3mGL (lacking the first 60 amino acid N-terminal domain in addition to the single point mutations in each GPSM motif) were generated by Dr. Peishen Zhao, a previous PhD student in our lab. Mutations in each GPSM motif were generated using a Site-Directed Mutagenesis Kit and then subclonded into the pCMV6a/b vector to generate Flag-tagged proteins as described in Zhao *et al.* 2010.

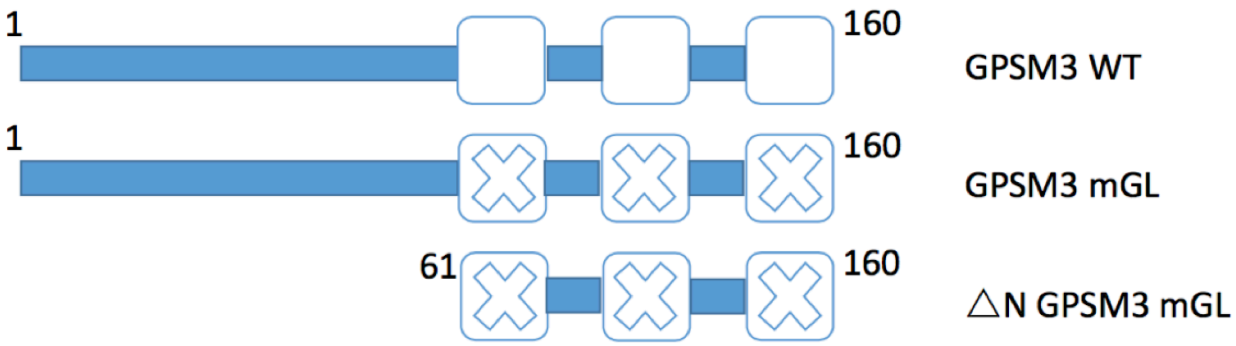

Figure 2-1. GPSM3 wildtype and mutant constructs. An arginine to phenylalanine single point mutation (represented as an "X") was induced in the last amino acid of each GPSM motifs. These mutations are denotated by mGL (mutated GoLoco or GPSM). In addition, the first 60 amino acids of the N-terminal domain were truncated and the mutation is labelled as ΔN as described in *Materials and Methods.*

2.7 *Gα$_i$ INACTIVATION VIA PERTUSSIS TOXIN*

cAMP MEASUREMENT

HEK-293H cells were transiently transfected with GloSensor™ cAMP biosensor, α_{2A}-adrenergic receptor and Flag-GPSM3 or Mock Vector plasmids as described under *Transfection Conditions*. Cells were then plated at a seeding density of 5 x 10^4 cells/well (1.5 x 10^5 cells/cm^2) on solid white bottom 96-well plates (Corning) and incubated at 37°C and 5% CO_2 for 4 hours to allow cells to sufficiently attach to well bottoms. Following this incubation period, 10 µl of medium was removed from each well and substituted with 10 µl of pertussis toxin at a concentration of 200 ng/ml or vehicle for 18 hours. The following day, medium was aspirated and replaced with 70 µl of fresh phenol red-free MEM, supplemented with 2 mM D-luciferin, 20 mM HEPES and 0.1% BSA (w/v) (pH = 7.20 ± 0.02; 300 ± 5 mOsmol/L). Cells were pretreated with 400 mM of IBMX and then treated with UK 14,304 (10 fM-1 nM) or vehicle (0.05% DMSO in PBS) 15 mins later. After a 5-min incubation with UK 14,304, cells were stimulated with DMB-Forskolin (final concentration of 2 µM). Luminescence intensity, which equates to the level of cytosolic cAMP, was measured from live cells every 90s.

β-ARRESTIN RECRUITMENT

HEK-293H cells were transfected with modified forms of α_{2A}-adrenergic receptor and β-arrestin-1 in the presence and absence of Flag-GPSM3, as described under *Transfection Conditions*. Cells were then plated at a seeding density of 5 x 10^4 cells/well (1.5 x 10^5 cells/cm^2) on solid white bottom 96-well plates (Corning) and incubated at 37°C and 5% CO_2 for 4 hours to allow cells to sufficiently attach to well bottoms. Following this incubation, 10 µl of medium

was removed from each well and substituted with 10 μl of pertussis toxin at a concentration of 200 ng/ml or vehicle for 18 hours. The following day, medium was removed and replaced with 90 μl of fresh phenol red-free MEM (supplemented with 3.2 mM D-luciferin, 20 mM HEPES, and 0.1% BSA; pH = 7.20 ± 0.02; 300 ± 5 mOsmol/L). Cells were then incubated for one hour at 37°C. Following incubation, cells were stimulated with UK 14,304 (10 fM – 10 μM). Luminescence was measured using LMax^{TM}II^{384} for 80 mins at 37°C with an integration time of 2 seconds and an interval of 2 minutes.

2.8 *DATA ANALYSES AND STATISTICS*

Data shown are represented as means ± SEM. Differences between two groups were assessed using a Student's *t*-test. Differences between more than two groups were determined using One-Way or Two-Way ANOVA, followed by a Bonferroni multiple comparisons test. Live cell cAMP measurements and β-arrestin-1-receptor interactions were collected as luminescence *versus* time curves. Average slope was calculated from each curve by calculating the mean of five consecutive data points and then normalizing each slope to the highest overall slope within that experiment, as described by Kim *et al.* 2018 (Figure 2-2). Concentration dose-response data was fitted using GraphPad Pad Prism 6.2 software (La Jolla, CA) to a 3-parameter sigmoidal equation using a consecutive non-linear regression analysis of multiple data sets. Three-parameters (minimum signal, EC_{50} or IC_{50}, and maximum signal) were analyzed individually to establish differences between curves. An extra sum-of-squares *F*-test was used to analyze the effect of GPSM3 on all three parameters studied. A *p* value of less than 0.05 was accepted as statistically significant.

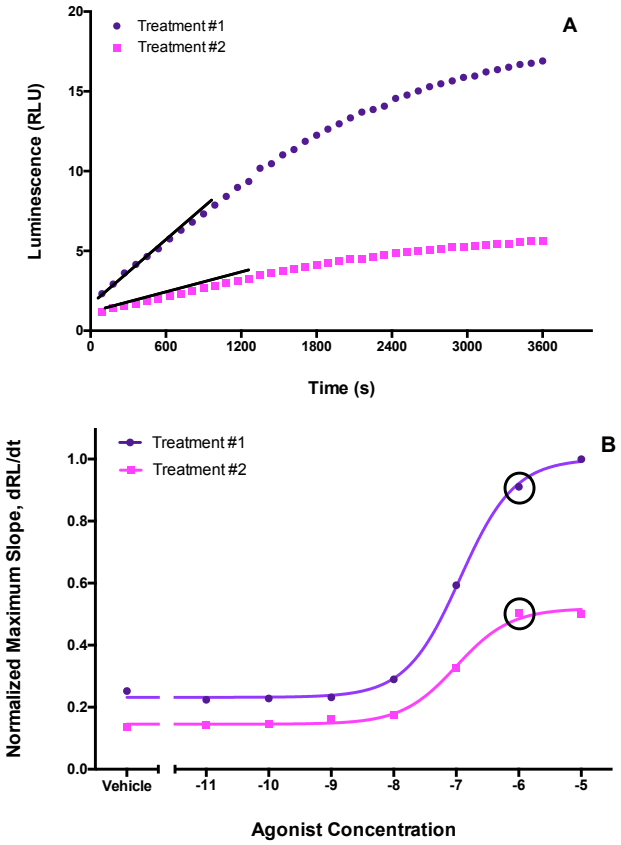

Figure 2-2. Determination of maximal β-arrestin recruitment and adenylyl cycle activity from luminescence vs time data. HEK-293H cells were transiently transfected with either GloSensor™ cAMP biosensor plasmid for cAMP detection or co-transfected with plasmids encoding N-terminal luciferase fragment-β-arrestin-1 and C-terminal luciferase fragment GPCR for detection of β-arrestin-1 recruitment to the receptor, as described in *Materials and Methods.* Panel (**A**) illustrates luminescence intensity, which corresponds to either the level of β-arrestin-1-receptor interaction or cAMP measured in a time-dependent manner. β-arrestin-1-receptor interaction or adenylyl cyclase activity was determined from each curve by measuring the greatest slope of each individual curve. Data was then normalized and represented as a fraction of the greatest rate of luminescence within each experiment, as shown in panel (**B**). Concentration dependence curves were fitted to a 3-parameter sigmoidal equation using nonlinear regression. Data points corresponding to the slopes shown in panel (**A**) are indicated with *black circles* in panel (**B**).

Chapter 3

3 RESULTS

3.1 *GPSM3 REDUCES BINDING OF β-ARRESTIN-1 TO α_{2A}-ADRENERGIC AND μ-OPIOID RECEPTORS*

I first investigated the effect of GPSM3 overexpression on the recruitment of β-arrestin-1 to G_i-coupled receptors including α_{2A}-adrenergic and μ-opioid receptors. The recruitment of β-arrestin-1 to each receptor was monitored in real-time using a luciferase complementation assay (Misawa *et al.* 2010). HEK293H cells, a human embryonic kidney cell line that does not express GPSM3 endogenously (The Human Protein Atlas, 2018), were transiently transfected with plasmids encoding the biosensor in addition to Flag-GPSM3 or control. The following day, cells transfected with α_{2A}-adrenergic receptor were stimulated with the agonist UK 14,304 (10 pM – 10 μM or vehicle) and cells with μ-opioid were stimulated with the agonist DAMGO (100 pM – 100 μM or vehicle). Luminescence was measured over 80 mins. The rate of luminescence increase is proportional to the rate of β-arrestin-1 recruitment.

Results indicate that basal and maximal rates of β-arrestin-1 recruitment to α_{2A}-adrenergic receptor are reduced with wild type Flag-GPSM3 by 48.9% and 42.0%, respectively, when compared with control vector. Additionally, there was no change to EC_{50} when compared with control vector (Fig 3-1A). Conversely, basal and maximal rates of β-arrestin-1 recruitment to μ-opioid receptor are reduced with wild type Flag-GPSM3 by 27.1% and 54.4%, respectively, when compared with control vector. Additionally, there was no change to EC_{50} when compared with control vector (Fig 3-1B).

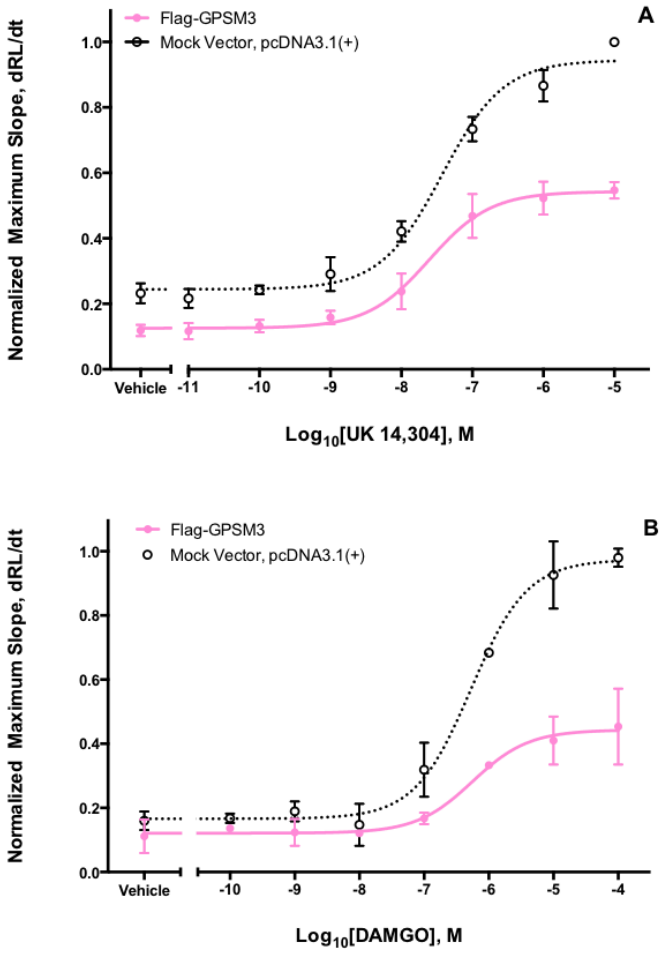

Fig. 3-1. GPSM3 reduces agonist-stimulated recruitment of β-arrestin-1 to G_i-coupled, α_{2A}-adrenergic and μ-opioid receptors. HEK293H cells were co-transfected with plasmids encoding N-terminal luciferase fragment-β-arrestin-1 and C-terminal luciferase fragment α_{2A}-adrenergic (**A**) or μ-opioid receptor (**B**) plus either GPSM3 (pink line) or control vector (dashed line). Cells were stimulated with indicated concentrations of UK 14,304 (**A**) or DAMGO (**B**). Values are represented as means ± SEM (n=3 independent experiments, each performed in duplicate), and fitted and compared as described under Materials and Methods. (**A**) $LogEC_{50}$ for UK 14,304 was equivalent between cells co-transfected either Flag-GPSM3-encoding plasmid (-7.6 ± 0.1) or Mock Vector (-7.43 ± 0.08), respectively. Upper asymptotic values differed significantly between

cells transfected with Flag-GPSM3-encoding plasmid (0.54 ± 0.01) vs. Mock Vector (0.94 ± 0.02) ($p < 0.0001$). Lastly, lower asymptotic values differed significantly between cells co-transected with Flag-GPSM3-encoding plasmid (0.13 ± 0.01) vs. Mock Vector (0.24 ± 0.01) ($p < 0.0001$) **(B)** $LogEC_{50}$ for DAMGO was equivalent between cells co-transfected with either Flag-GPSM3-encoding plasmid (-6.3 ± 0.2) or Mock Vector (-6.28 ± 0.08). Upper asymptotic values differed significantly between cells transfected with Flag-GPSM3-encoding plasmid (0.44 ± 0.03) vs. Mock Vector (0.97 ± 0.02) ($p < 0.0001$). Lastly, lower asymptotic values did not differ significantly between cells co-transected with Flag-GPSM3-encoding plasmid (0.12 ± 0.01) vs. Mock Vector (0.17 ± 0.01). All statistical values were based on extra sum of squares *F*-tests.

3.2 *GPSM3 HAS NO EFFECT ON THE BINDING OF β-ARRESTIN-1 TO G_s- OR $G_{q/11}$-COUPLED RECEPTORS*

Next, I evaluated whether GPSM3 selectively targeted G_i-coupled receptors. In order to do so, HEK293H cells were transiently transfected with β-arrestin-1 and either G_s-coupled receptors, β_2 adrenergic (β_2-AR) and parathyroid hormone type-1 (PTH1R), or the $G_{q/11}$-coupled muscarinic type-1 (M1) receptor together with either Flag-GPSM3 or control vector. Cells were then stimulated with receptor specific agonists and time-dependent changes in luminescence were measured as described above. Maximal rates were calculated for individual curves and then normalized as a percentage of the highest value in each individual experiment. The rates were measured in Relative Luminescent Units (RLU) per min of β-arrestin-1 recruitment to G_s- and $G_{q/11}$-coupled receptors did not differ significantly between GPSM3 and control-transfected cells. Thus, it appears that GPSM3 selectively reduces β-arrestin-1 recruitment to G_i-coupled receptors, with no effect on G_s- or $G_{q/11}$-coupled GPCRs.

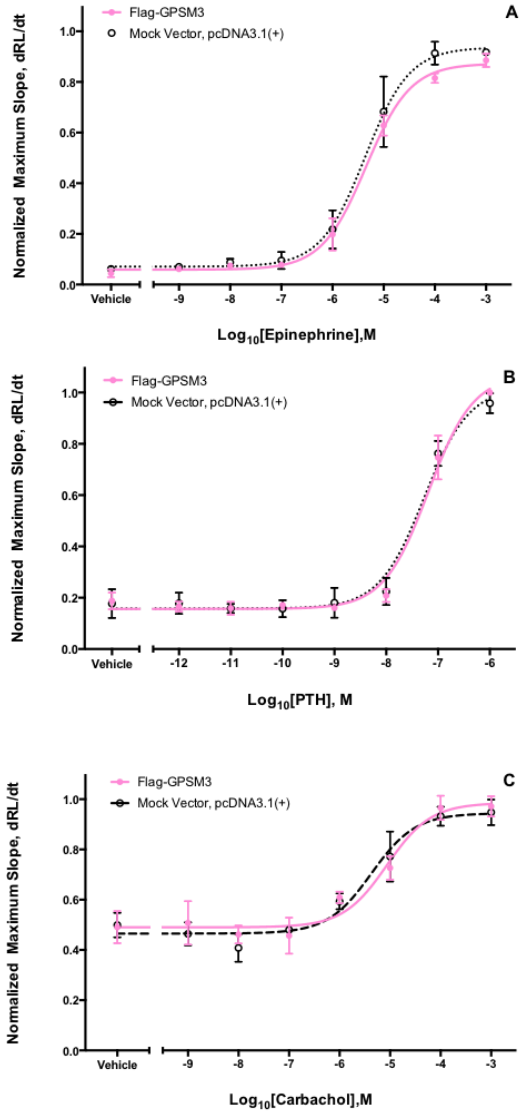

Fig. 3-2. GPSM3 does not affect agonist-stimulated recruitment of β-arrestin-1 to G_s-coupled β_2-adrenergic and parathyroid hormone type-1 nor $G_{q/11}$-coupled muscarinic type-1 receptors. HEK293H cells were co-transfected with plasmids encoding N-terminal luciferase fragment-β-arrestin-1 and C-terminal luciferase fragment β_2-adrenergic (**A**) parathyroid hormone type-1 (**B**) or muscarinic type-1 (**C**) plus either Flag-GPSM3 (pink line) or control vector (dashed line). Cells co-transfected with β_2-adrenergic receptor were stimulated with increasing concentrations of adrenaline/epinephrine or vehicle, whereas cells co-transfected with PTH1R were stimulated with PTH (1-34) or vehicle. Lastly, cells co-transfected with muscarinic type-1

receptor were stimulated with carbachol or vehicle. Values are represented as means ± SEM (n=3 independent experiments, each performed in duplicate). (**A**) $LogEC_{50}$ for adrenaline/epinephrine in cells expressing Flag-GPSM3 was -5.26 ± 0.04 and in control cells was -5.35 ± 0.06; these two values did not differ significantly, based on extra sum-of-squares *F*-test. (**B**) $LogEC_{50}$ for PTH (1-34) in cells co-transfected with Flag-GPSM3 was -7.2 ± 0.1 while in cells co-transfected with Mock Vector the $LogEC_{50}$ for PTH (1-34) was -7.3 ± 0.1; these two values did not differ significantly, based on extra sum-of-squares *F*-test. (**C**) The $LogEC_{50}$ for carbachol in cells co-transfected with Flag-GPSM3 was -5.06 ± 0.16 whereas in cells co-transfected with Mock Vector the fitted value was -5.37 ± 0.16; these two values did not differ significantly. All statistical values were based on extra sum of squares *F*-tests.

3.3 *N-TERMINAL REGION AND GPSM MOTIFS ARE BOTH REQUIRED FOR THE REDUCTION OF β-ARRESTIN-1 BINDING TO α_{2A} ADRENERGIC RECEPTOR*

I have established that GPSM3 can selectively decrease β-arrestin-1 recruitment to G_i-coupled receptors, with no measurable effect on G_s- or $G_{q/11}$-coupled GPCRs. As a result, I wanted to test which protein domains within GPSM3 are important for its effects on GPCR signalling. Structurally, GPSM3 is comprised of an N-terminal domain and three GPSM regions that are highly conserved among group II AGS proteins. A series of mutant GPSM3 constructs developed by Dr. Peishen Zhao were used to examine changes in β-arrestin-1 recruitment to α_{2A}-adrenergic receptor when compared with wild-type protein and control. HEK293H cells were transfected with plasmids encoding Flag-GPSM3, Flag-GPSM3mGL, Flag-ΔN•GPSM3mGL or control. Flag-GPSM3mGL contains single point mutations (Arg → Phe) in each of the three GPSM motifs whereas Flag-ΔN•GPSM3mGL is a quadruple mutant that lacks the N-terminal domain in addition to the aforementioned GPSM mutations.

Results indicate that basal and maximal rates of β-arrestin-1 recruitment to α_{2A}-adrenergic receptor are reduced with wild type Flag-GPSM3 by 43.2% and 46.9%, respectively, but only 24.5% and 21.9%, respectively, with the mutant Flag-GPSM3mGL. Furthermore, there was no measurable difference between cells co-transfected with Flag-ΔN•GPSM3mGL and control. This strongly suggests that the N-terminal domain and GPSM motifs both contribute to inhibition of β-arrestin-1 recruitment.

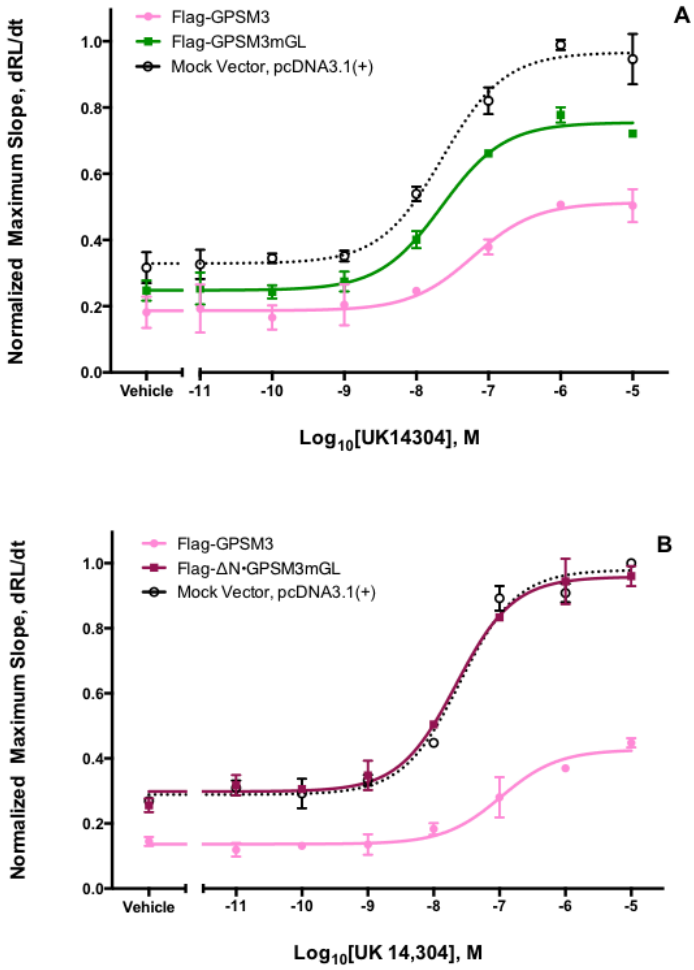

Fig. 3-3. N-terminus and GPSM motifs are both essential for reduced interaction between β-arrestin-1 and α_{2A} adrenergic receptor. HEK293H cells were co-transfected with plasmids encoding N-terminal luciferase fragment-β-arrestin-1 and C-terminal luciferase fragment α_{2A}-adrenergic receptor in the presence of wildtype (pink line), mutated versions of GPSM3; GPSM3mGL in *panel A* and ΔN•GPSM3mGL in *panel B* and Mock Vector (dashed line). Cells were stimulated with increasing concentrations of UK 14,304 or vehicle. Values are represented as means ± SEM (n=3 independent experiments, each performed in duplicate), and fitted and compared as described under Materials and Methods. (**A**) $LogEC_{50}$ for UK 14,304 was equivalent between cells co-transfected either Flag-GPSM3-encoding plasmid (-7.21 ± 0.09), Flag-GPSM3mGL-encoding plasmid (-7.65 ± 0.08) and Mock Vector (-7.64 ± 0.07). Upper asymptotic values differed significantly between cells transfected with Flag-GPSM3-encoding plasmid (0.51

± 0.02), Flag-GPSM3mGL-encoding plasmid (0.75 ± 0.01), vs. Mock Vector (0.96 ± 0.01) ($p <$ 0.0001). Lower asymptotic values differed significantly between cells co-transected with Flag-GPSM3-encoding plasmid (0.18 ± 0.01), Flag-GPSM3mGL-encoding plasmid (0.24 ± 0.01) vs. Mock Vector (0.33 ± 0.01) ($p < 0.0001$) (**B**) $LogEC_{50}$ for UK 14, 304 was equivalent for cells transfected with either Flag-GPSM3-encoding plasmid (-6.98 ± 0.13), Flag-ΔN•GPSM3mGL-encoding plasmid (-7.66 ± 0.07) and Mock Vector (-7.60 ± 0.08). Upper asymptotic values differed significantly between cells transfected with Flag-GPSM3-encoding plasmid (0.43 ± 0.01) vs. Mock Vector (0.98 ± 0.02) ($p < 0.0001$) but not with Flag-ΔN•GPSM3mGL-encoding plasmid (0.96 ± 0.01) vs. Mock Vector. Lastly, lower asymptotic values differed significantly between cells transfected with Flag-GPSM3-encoding plasmid (0.14 ± 0.01) vs. Mock Vector (0.29 ± 0.01) ($p <$ 0.0001), but not between Flag-ΔN•GPSM3mGL-encoding plasmid (0.30 ± 0.01) vs. Mock Vector. All statistical values were based on extra sum of squares *F*-tests.

3.4 ACTIVATION OF Gαi IS NOT NECESSARY FOR INHIBITORY GPSM3 EFFECT ON β-ARRESTIN-1 BINDING TO α_{2A}-ADRENERGIC RECEPTOR

My next goal was to evaluate the process through which GPSM3 functions. Canonically, GPSM3 binds to and promotes the GDP-bound form of $G\alpha_i$, while subsequently preventing $G\beta\gamma$ re-association to the heterotrimeric complex. To assess whether $G\alpha_i$ protein activation is a necessary step for GPSM3 to reduce β-arrestin-1 recruitment, I decided to pre-treat cells with pertussis toxin, a potent biotoxin that inactivates $G\alpha_i$ proteins.

To test the viability of pertussis toxin, HEK293H cells were first transfected with a plasmid encoding a luciferase-based cAMP biosensor, α_{2A}-adrenergic receptor and Flag-GPSM3 or control. Cells were then pre-treated with pertussis toxin (200 ng/ml) or vehicle for 18-hours until sequential addition of IBMX (400 mM), UK 14,304 (1 fM – 1 nM) and DMB-forskolin (2 μM final) as described in *Materials and Methods*. It is well established that cells treated with pertussis toxin lose their ability to inhibit adenylyl cyclase activity (Mangmool *et al.* 2011), and indeed pertussis toxin treatment unmasked the ability of α_{2A}-adrenergic receptor to activate G_s signalling (Figure 3-4A) as demonstrated previously by (Eason *et al.* 1992). Conversely, in α_{2A}-adrenergic receptor-expressing cells treated with vehicle rather than PTX, the ability of UK 14, 304 to inhibit cAMP production was maintained. This confirms that the toxin was in fact viable and successfully inhibited activation of $G\alpha_i$ proteins.

I then assessed whether $G\alpha_i$ inactivation via pertussis toxin could affect the ability of GPSM3 to reduce β-arrestin-1 recruitment to the α_{2A} adrenergic receptor. Cells were transfected with plasmids encoding β-arrestin-1, α_{2A}-adrenergic receptor and Flag-GPSM3 or control vector,

and subsequently treated cells with pertussis toxin (200 ng/ml) or vehicle for 18-hours. The following day, cells were stimulated with UK 14,304 (10 pM – 10 μM). There was no measureable change in β-arrestin-1 recruitment to the α_{2A}-adrenergic receptor in cells (transfected with Flag-GPSM3) treated with pertussis toxin as compared to vehicle. Furthermore, there was no change β-arrestin-1 recruitment in cells (transfected with control) treated with pertussis toxin and vehicle. This observation implies that activation of $G\alpha_i$ is not required for the inhibitory effect of GPSM3 on the recruitment of β-arrestin-1to the receptor.

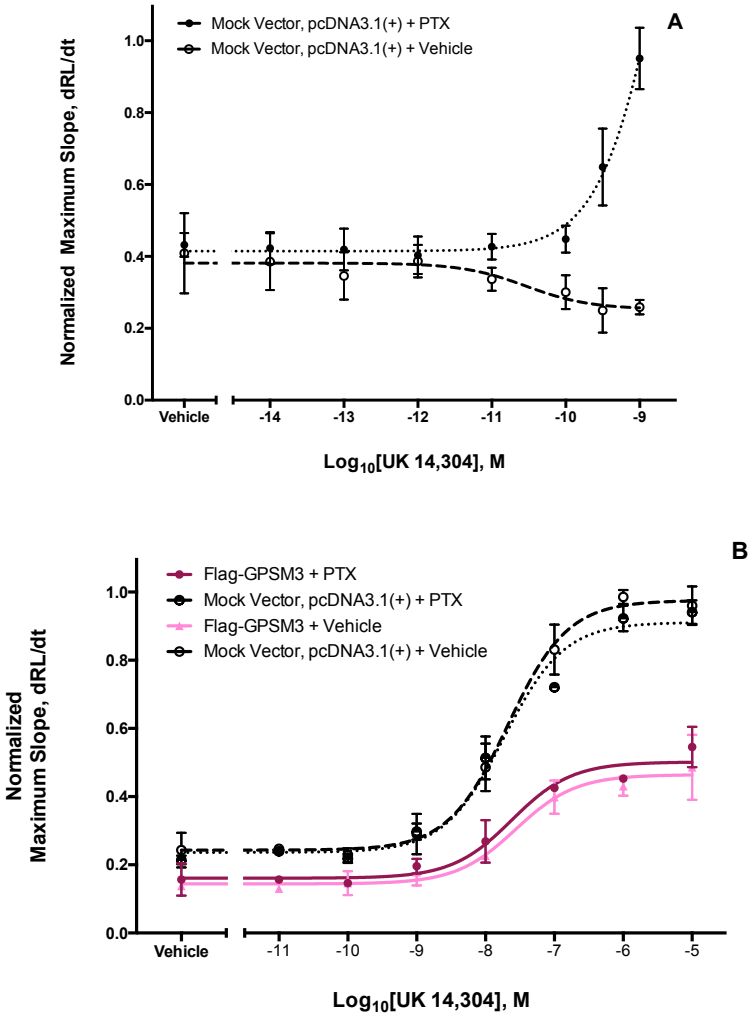

Fig. 3-4. Pertussis toxin does not affect GPSM3's ability to inhibit β-arrestin-1 recruitment to α_{2A}-adrenergic receptor. (**A**-**B**) HEK293H cells were transfected with GloSensor™ cAMP biosensor, α_{2A}-adrenergic receptor and Flag-GPSM3 or Mock Vector plasmids. Cells were treated with pertussis toxin (PTX) (200 ng/ml) for 18 hours prior to the addition of agonist as discussed in *Materials and Methods*. (**A**) Illustrates dose-response curves of cells treated with pertussis toxin (dotted line) or vehicle (dashed line). Data were normalized as a fraction of the greatest value of cyclase activity in each experiment. Values are represented as means ± SEM (n = 3 independent experiments, each performed in triplicate). (**B**) HEK293H cells were co-transfected with plasmids encoding N-terminal luciferase fragment-β-arrestin-1 and C-terminal luciferase fragment α_{2A}-adrenergic receptor in the presence and absence of GPSM3. Cells were pretreated with pertussis

toxin or vehicle for 18 hours and then stimulated with UK 14,304 (10fM – 10μM) or vehicle. Maximal rate of β-arrestin-1-receptor interaction was determined and normalized as previously discussed. Values are represented as means ± SEM (n=3 independent experiments, each performed in duplicate). $LogEC_{50}$ values for UK 14,304 did not differ between PTX-treated cells transfected with Flag-GPSM3 (-7.619 ± 0.16) vs. Mock Vector (-7.730 ± 0.1), nor did values for vehicle-treated cells transfected with Flag-GPSM3 (7.562 ± 0.17) vs. Mock Vector (7.678 ± 0.08). Upper asymptotic values differed significantly for PTX-treated cells transfected with Flag-GPSM3 (0.5016 ± 0.01) vs. Mock Vector (0.9110 ± 0.02) ($p < 0.001$) and for vehicle-treated cells transfected with Flag-GPSM3 (0.4643 ± 0.01) vs. Mock Vector (0.9753 ± 0.02) ($p < 0.001$). In addition, differences between PTX and vehicle groups were not significant. Lastly, lower asymptotic values differed significantly for PTX-treated cells transfected with Flag-GPSM3 (0.1608 ± 0.01) vs. Mock Vector (0.2364 ± 0.01) ($p < 0.001$) and for vehicle-treated transfected with Flag-GPSM3 (0.1440 ± 0.01) vs. Mock Vector (0.2432 ± 0.01) ($p < 0.001$). In addition, differences between PTX and vehicle groups were not significant. All statistical values were based on extra sum of squares *F*-tests.

3.5 $G\beta_1\gamma_2$ OVEREXPRESSION REDUCES β-ARRESTIN-1 BINDING TO α2A-ADRENERGIC RECEPTOR

To further interrogate the mechanism by which GPSM3 impedes β-arrestin recruitment, I evaluated the impact of $G\beta_1\gamma_2$ overexpression. In addition to binding to and inhibiting activity of $G\alpha_i$, GPSM3 promotes $G\beta_1\gamma_2$ signalling by preventing its re-association to $G\alpha_i$. At the same time, GPSM3 and $G\beta_1\gamma_2$ compete with each other for binding to $G\alpha_i$. To study the impact of $G\beta_1\gamma_2$ signalling, HEK293H cells were transfected with plasmids encoding β-arrestin-1 and α_{2A}-adrenergic receptor fusion proteins (modified to include complementary luciferase domains), as well as Flag-GPSM3 or control vector, and $G\beta_1\gamma_2$ or control vector. Maximal rates of recruitment were calculated from raw time course data.

Results indicate that basal and maximal rates of β-arrestin-1 recruitment to α_{2A}-adrenergic receptor are reduced with $G\beta_1\gamma_2$ overexpression alone by 37.1% and 47.5%, respectively, when compared with control vector. This change was comparable to that of Flag-GPSM3, which reduced basal and maximal rates of β-arrestin-1 recruitment to α_{2A}-adrenergic receptor by 37.0% and 41.3%, respectively, when compared with control. Moreover, $G\beta_1\gamma_2$ co-transfected with Flag-GPSM3 had an additive effect and reduced basal and maximal β-arrestin-1 recruitment to α_{2A}-adrenergic receptor by 64.7% and 66.6%, respectively, when compared with control. Additionally, there was no change to EC_{50} of UK 14,304 in cells transfected with $G\beta_1\gamma_2$ alone, Flag-GPSM3 alone or cells co-transfected with both $G\beta_1\gamma_2$ and Flag-GPSM3 when compared with control vector (Fig 3-5).

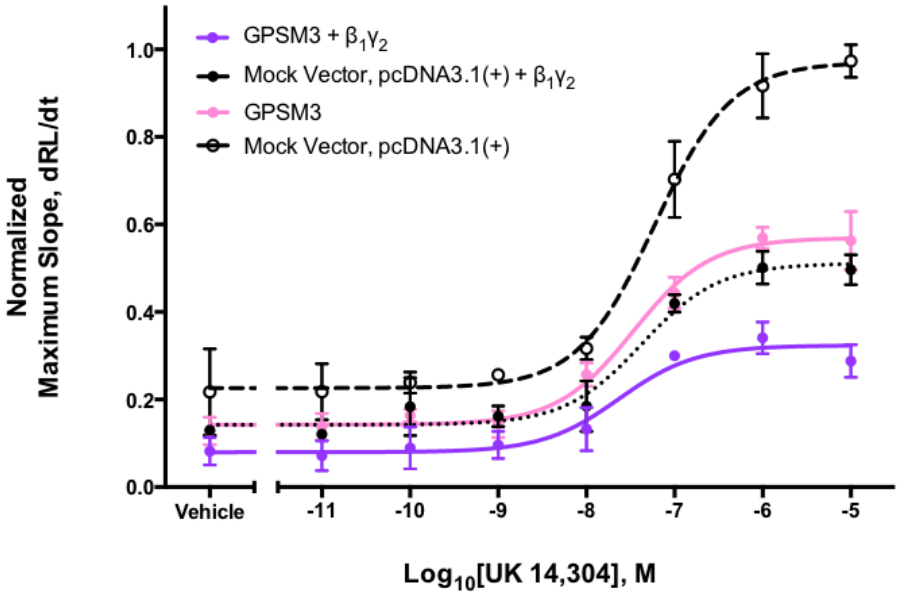

Fig. 3-5. Overexpression of Gβ$_1$γ$_2$ reduces recruitment of β-arrestin-1 to α$_{2A}$-adrenergic receptor in the presence or absence of GPSM3. HEK293H cells were co-transfected with plasmids encoding N-terminal luciferase fragment-β-arrestin-1, C-terminal luciferase fragment α$_{2A}$ adrenergic, Flag-GPSM3 or control vector in the presence or absence of Gβ$_1$γ$_2$ in each group. Cells transfected were stimulated with UK 14,304 (10pM – 10μM) or vehicle (0.05% DMSO in PBS). Values are represented as means ± SEM (n=3 independent experiments, each performed in duplicate). LogEC$_{50}$ values for UK 14,304 for cells co-transfected with Flag-GPSM3 and Mock Vector transfected with Gβ$_1$γ$_2$ were -7.63 ± 0.18 and -7.37 ± 0.13, respectively (p>0.05). Conversely, LogEC$_{50}$ values for UK 14, 304 did not differ between cells transfected with Flag-GPSM3 (-7.47 ± 0.09) vs. Mock Vector (-7.23 ± 0.08) (p<0.05). Upper asymptotic values differed between cells transfected with Flag-GPSM3 + Gβ$_1$γ$_2$ (0.32 ± 0.01) vs. Mock Vector + Gβ$_1$γ$_2$ (0.51 ± 0.01) (p < 0.0001). Conversely, LogEC$_{50}$ values for UK 14,304 differed between cells transfected with Flag-GPSM3 + control (0.57 ± 0.01) vs. Mock Vector + control (0.97 ± 0.02) (p < 0.0001). Lastly, bottom asymptotic values differed between cells transfected with Flag-GPSM3 + Gβ$_1$γ$_2$ (0.08 ± 0.01) vs. Mock Vector + Gβ$_1$γ$_2$ (0.14 ± 0.01) (p < 0.0001). Furthermore, bottom asymptotic values differed between cells transfected with Flag-GPSM3 + control (0.14 ± 0.01) vs. Mock Vector + control (0.23 ± 0.01) (p < 0.0001). All statistical values were based on extra sum of squares F-tests.

3.6 REDUCTION OF β-ARRESTIN-1 BINDING TO α_{2A}-ADRENERGIC RECEPTOR WITH GPSM3 IS RESCUED WITH GRK2CT

As shown in Figure 3-5, $G\beta_1\gamma_2$ overexpression alone was capable of reducing recruitment of β-arrestin-1 to α_{2A}-adrenergic receptor. Moreover, the reduction seen with GPSM3 was exacerbated by the further addition of $G\beta_1\gamma_2$. I next investigated whether treatment with GRK2ct could reverse these effects. GRK2ct is the C-terminal portion of GRK2 that binds to the Gβ subunit within the Gβγ dimer. As a result, GRK2ct is frequently used as a Gβγ inhibitor to study Gβγ-dependent signalling pathways (Nadella *et al.* 2010). HEK293H cells were transfected with plasmids encoding β-arrestin-1 and α_{2A}-adrenergic receptor fusion proteins (modified to include complementary luciferase domains), together with either Flag-GPSM3 or control vector plus either GRK2ct or additional control vector. GRK2ct alone did not affect β-arrestin-1 recruitment to α_{2A}-adrenergic receptor when compared with control. However, the reduction in β-arrestin-1 recruitment seen with GPSM3 was reversed in cells additionally expressing GRK2ct. Thus, the rescue effect observed with GRK2ct, a Gβγ-specific inhibitor, strongly suggests that the mechanism by which GPSM3 functions is through a Gβγ-dependent mechanism.

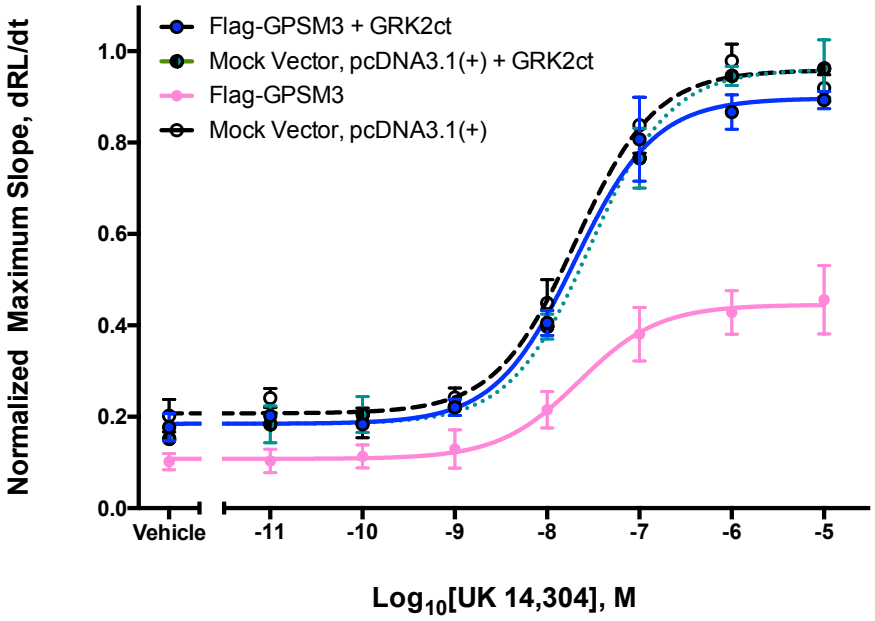

Fig. 3-6. GRK2ct rescues the reduction of β-arrestin-1 binding to α_{2A} adrenergic receptor by GPSM3. HEK293H cells were co-transfected with plasmids encoding N-terminal luciferase fragment-β-arrestin-1 and C-terminal luciferase fragment α_{2A}-adrenergic receptor, together with Flag-GPSM3 or control vector and also GRK2ct ($G\beta_1\gamma_2$ inhibitor), or its control vector. Cells transfected were stimulated with UK 14,304 (10pM – 10μM) or vehicle (0.05% DMSO in PBS). Values are represented as means ± SEM (n=3 independent experiments, each performed in duplicate). $LogEC_{50}$ values for UK 14,304 did not differ between cells transfected with GRK2ct + Flag-GPSM3 (-7.70 ± 0.05) vs. GRK2ct + control (-7.54 ± 0.05). Conversely, $LogEC_{50}$ values for UK 14,304 did not differ between cells transfected with control + Flag-GPSM3 (-7.66 ± 0.05) vs. control + control (-7.69 ± 0.08). In addition, differences in $LogEC_{50}$ values for 14, 304 were not significant between GRK2ct and vehicle groups. Upper asymptotic values differed between cells transfected with GRK2ct in the presence (0.90 ± 0.01) and absence (0.96 ± 0.01) of GPSM3 ($p < 0.0001$). Upper asymptotic values also differed between cells lacking GRK2ct in the presence (0.445 ± 0.005) and absence (0.96 ± 0.02) of Flag-GPSM3 ($p < 0.0001$). Lastly, lower asymptotic values did not differ between cells transfected GRK2ct in the presence (0.185 ± 0.009) and absence (0.18 ± 0.01) of Flag-GPSM3. Finally, basal fitted values differed between cells lacking GRK2ct in the presence (0.108 ± 0.004) and absence (0.21 ± 0.01) of Flag-GPSM3 ($p < 0.0001$). All statistical values were based on extra sum of squares *F*-tests.

3.7 INHIBITION OF cAMP BY Gαi-COUPLED RECEPTORS IS ALTERED IN THE PRESENCE OF GPSM3

I next investigated the potential effect of GPSM3 on the abilities of G_i-coupled GPCRs to inhibit adenylyl cyclase activity. To do this, I studied the effects using three different receptors: adenosine A1, α_{2A}-adrenergic and μ-opioid. HEK293H cells were transfected with a luciferase based cAMP biosensor and receptor of interest in the presence or absence of GPSM3. To prevent cAMP degradation, cells were treated with cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX). Cells were then treated with a receptor-specific agonist or vehicle; cells transiently expressing adenosine A1 were treated with 2-chloro-N(6) cyclopentyladenosine (CCPA) (10 pM – 10 μM) whereas cells with α_{2A}-adrenergic and μ-opioid were treated with UK 14,304 (10 fM – 1 nM) and [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO) (100 pM – 100 μM), respectively. Finally, cells were stimulated with 7-Deacetyl-7-[O-(N-methylpiperazino)-γ-butyryl]-Forskolin Dihydrochloride (DMB-Forskolin) (2 μM final), which directly stimulates adenylyl cyclase activity.

In the absence of agonist, cells overexpressing adenosine A1, α_{2A}-adrenergic and μ-opioid receptors all exhibited a marked reduction in basal DMB-forskolin-stimulated cAMP production when transfected with GPSM3 by 24.7%, 26.3% and 21.8%, respectively. Furthermore, there was no change in IC_{50} values between GPSM3 and control groups in all receptors except μ-opioid, which had a rightward shift with GPSM3 ($LogIC_{50}$=-7.49) compared to control ($LogIC_{50}$=-7.91). Lastly, bottom asymptotic values did not differ between GPSM3 and control groups in all receptors, except for μ-opioid, which had a marked increase in inhibition by

16.26% with GPSM3 compared to control. Therefore, presence of GPSM3 significantly reduces basal forskolin-stimulated cAMP production in G_i-coupled GPCR, although the mechanism by which this occurs is unknown.

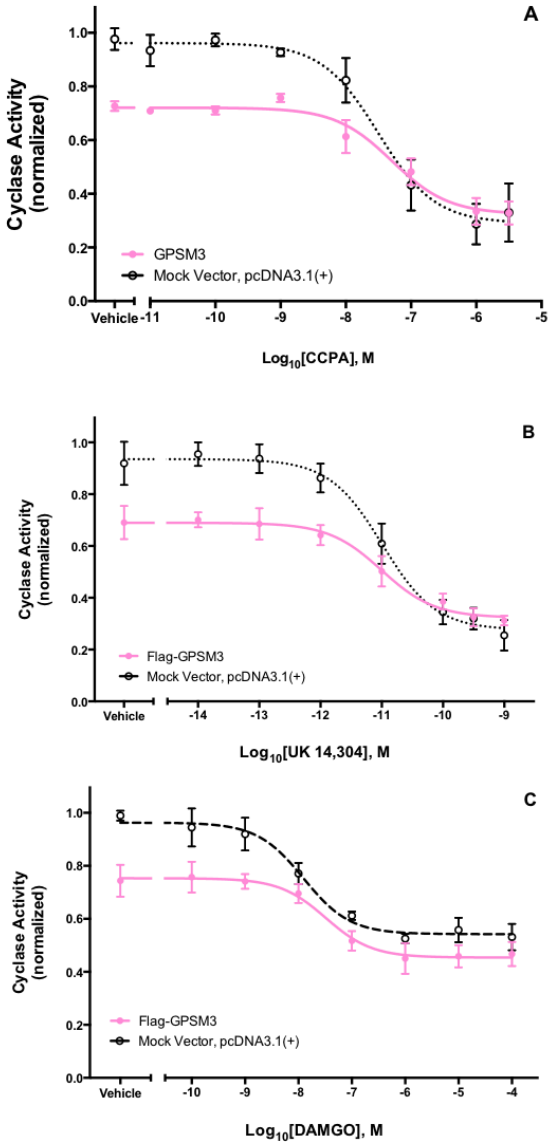

Fig. 3-7. GPSM3 decreases basal forskolin-stimulated cAMP production.
HEK293H cells were co-transfected with GloSensor™ cAMP biosensor plasmid and either adenosine A1 (**A**), α_{2A}-adrenergic (**B**), or μ-opioid (**C**) receptor plasmids in the presence (pink line) or absence (dashed line) of GPSM3. Cells were pretreated with 400 mM IBMX and then treated with CCPA (**A**), UK 14,304 (**B**), DAMGO (**C**) or their vehicles 15 mins later. After a 5-min incubation with agonists, cells were stimulated DMB-Forskolin (final concentration of 2 μM). Luminescence intensity, which equates to the level of cytosolic cAMP, was measured from live cells every 90s. Data were normalized as a fraction of the greatest value of cyclase activity in each experiment as described in *materials and methods.* Values plotted are means ± SEM (*n* =

3 independent experiments, each performed in duplicate). (**A**) $LogEC_{50}$ for CCPA was equivalent between cells co-transfected with either Flag-GPSM3-encoding plasmid (-7.2 ± 0.2) or Mock Vector (-7.51 ± 0.16) (p=0.47). Upper asymptotic values differed significantly between cells transfected with Flag-GPSM3-encoding plasmid (0.72 ± 0.02) vs. Mock Vector (0.96 ± 0.02) ($p < 0.0001$). Lastly, lower asymptotic values did not differ significantly between cells co-transected with Flag-GPSM3-encoding plasmid (0.32 ± 0.03) vs. Mock Vector (0.29 ± 0.04) (p=0.61). (**B**) $LogEC_{50}$ for UK 14,304 was equivalent between cells co-transfected with either Flag-GPSM3-encoding plasmid (-11.00 ± 0.13) or Mock Vector (-10.99 ± 0.10) (p=0.95). Upper asymptotic values differed significantly between cells transfected with Flag-GPSM3-encoding plasmid (0.69 ± 0.01) vs. Mock Vector (0.93 ± 0.02) ($p < 0.0001$). Lastly, lower asymptotic values did not differ significantly between cells co-transected with Flag-GPSM3-encoding plasmid (0.32 ± 0.02) vs. Mock Vector (0.28 ± 0.02) (p=0.10). (**C**) $LogEC_{50}$ for DAMGO differed between cells co-transfected with either Flag-GPSM3-encoding plasmid (-7.49 ± 0.17) or Mock Vector (-7.91 ± 0.11). Upper asymptotic values differed significantly between cells transfected with Flag-GPSM3-encoding plasmid (0.75 ± 0.01) vs. Mock Vector (0.96 ± 0.02) ($p < 0.0001$). Lastly, lower asymptotic values differed significantly between cells co-transected with Flag-GPSM3-encoding plasmid (0.45 ± 0.01) vs. Mock Vector (0.54 ± 0.01) ($p < 0.0001$). All statistical values were based on extra sum of squares F-tests.

3.8 *GPSM3 HAS NO EFFECT ON THE STIMULATION OF cAMP PRODUCTION BY Gs-COUPLED RECEPTORS*

To further explore the potential effects of GPSM3 on adenylyl cyclase regulation, I examined the effect of GPSM3 on G_s-coupled β_2 adrenergic and parathyroid hormone type-1 receptors. Endogenous β_2-adrenergic receptor activity was studied in HEK293H cells, while the parathyroid hormone type-1 receptor was transiently transfected. To prevent cAMP degradation, cells were treated with cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX). Cells were then treated with a receptor-specific agonist or vehicle; cells co-transfected with β_2 adrenergic receptor were treated with adrenaline/epinephrine (100 pM – 100 μM) and cells co-transfected with parathyroid hormone type-1 receptor were treated with PTH (1-34) (1 pM – 1 μM). As predicted from its established G protein selectivity (Giguere *et al.* 2013, Kimple *et al.* 2004, Zhao *et al.* 2015), GPSM3 showed no measureable influence on G_s-coupled GPCR activation of adenylyl cyclase, with agonist dose-response curves being virtually superimposable in cells expressing GPSM3 vs mock vector controls. This result is consistent with GPSM3's ability to selectively bind to $G\alpha_i$ proteins but not $G\alpha_s$ proteins.

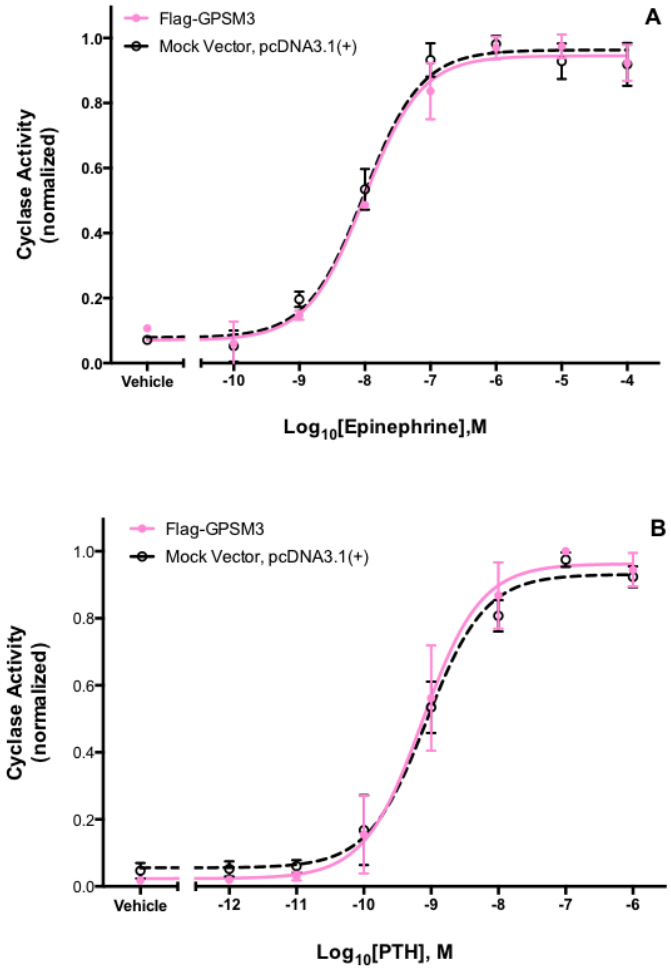

Fig. 3-8. Lack of GPSM3 effect on β_2-adrenergic and parathyroid hormone type-1 receptor-stimulated adenylyl cyclase activity. HEK293H cells were transfected with GloSensor™ cAMP biosensor as described in *Materials and Methods*. Receptor activity was measured with endogenous β_2-adrenergic receptor (**A**) or with transfected PTH1R (**B**) in the presence of GPSM3 or control. Cells were treated with IBMX (400 μM) for 20 mins and then stimulated with adrenaline/epinephrine (100 pM – 100 μM) or PTH (1-34) (1 pM – 1 μM). Data were normalized as a fraction of the greatest value of cyclase activity in each experiment. Values plotted are means ± SEM (n = 3 independent experiments, each performed in duplicate). (A) $LogEC_{50}$ values for epinephrine did not differ between cells transfected with Flag-GPSM3 (-7.92 ± 0.06) vs. control (-8.09 ± 0.08) (p=0.1239). Upper asymptotic values did not differ between cells transfected with Flag-GPSM3 (0.95 ± 0.02) vs. control (0.96 ± 0.02) (p=0.9452). Lastly, lower asymptotic values

did not differ between cells transfected with Flag-GPSM3 (0.07 ± 0.02) vs. control (0.07 ± 0.03) (p=0.79). (B) $LogEC_{50}$ values for PTH(1-34) did not differ between cells transfected with Flag-GPSM3 (-9.13 ± 0.09) vs. control (-9.07 ± 0.07) (p=0.59). Upper asymptotic values did not differ between cells transfected with Flag-GPSM3 (0.96 ± 0.03) vs. control (0.93 ± 0.02) (p=0.35). Lastly, lower asymptotic values did not differ between cells transfected with Flag-GPSM3 (0.02 ± 0.02) vs. control (0.05 ± 0.02) (p=0.27). All statistical values were based on extra sum of squares F-tests.

3.9 *FORSKOLIN POTENCY ON ENDOGENOUS ADENYLYL CYCLASE ACTIVTY IS DECREASED IN HEK293H CELLS OVEREXPRESSING GPSM3*

Next, I wanted to investigate the mechanism of GPSM3-medicated reduction in basal forskolin-stimulated but not G_s-stimulated adenylyl cyclase activty. To do this, I examined the effect of GPSM3 on endogenous adenyly cyclase activity within HEK293H cells. As in previous experiements, cells were co-transfected with a cAMP biosensor plus either control vector or GPSM3. In this experiment, cells were stimulated with various concentrations of DMB-forskolin (100 pM – 100 μM) or its vehicle (PBS + 0.1% BSA). Adenylyl cyclase acitivity was determined from time course data as a fraction of the maximal slope in each indepdendent experiment. In the presence of GPSM3, the DMB-forskolin concentration dependence curve was shifted to the right ($LogEC_{50}$ = -5.26 ± 0.03) when compared to the curve with control ($LogEC_{50}$ = -5.92 ± 0.04). This corresponds to an approximate 4.6-fold decrease in the measured potency.

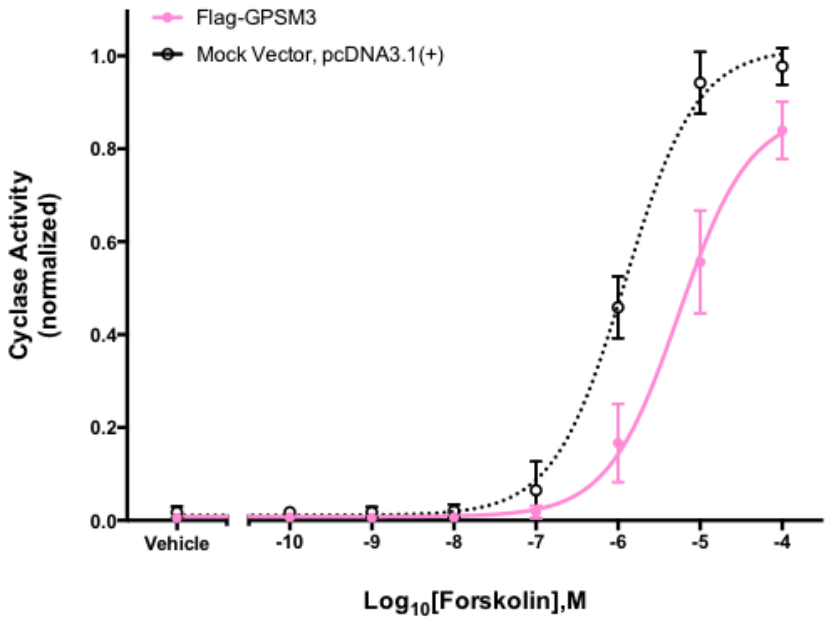

Fig. 3-9. GPSM3 decreases potency of DMB-forskolin on endogenous adenylyl cyclase activity in HEK293H cells. HEK293H cells were transfected with GloSensor™ cAMP biosensor as described in *Materials and Methods*. Receptor activity was measured with endogenous adenylyl cyclase activity in the presence of GPSM3 (pink line) or control (dashed line). Cells were treated with IBMX (400 μM) for 20 mins and then stimulated with DMB-Forskolin (100 pM – 100 μM) or vehicle. Data were normalized as a fraction of the greatest value of cyclase activity in each experiment. Values shown are means ± SEM (n = 3 independent experiments, each performed in duplicate). $LogEC_{50}$ values for DMB-forskolin in the presence and absence of Flag-GPSM3 and control were -5.26 ± 0.03 and -5.92 ± 0.04, respectively; representative curves demonstrate that GPSM3 reduces the potency of DMB-forskolin when compared with control ($p < 0.001$). All statistical values were based on extra sum of squares F-tests.

3.10 *GPSM3 ALTERS ADENYLYL CYCLASE ISOFORMS III AND VI BY SELECTIVELY INHIBITING $G\alpha_I$ ACTIVITY WITH NO EFFECT TO $G\alpha_I$-INSENSITIVE ISOFORM VI*

As previously mentioned, GPSM3 reduces DMB-forskolin stimulated adenylyl cyclase activity in HEK293H cells (Figure 3-9). Since HEK293H cells express multiple adenylyl cyclase isoforms with different Gα and Gβγ sensitivities, I next sought to perform experiments to investigate effects of GPSM3 on individual isoforms of adenylyl cyaclase. HEK293H cells endogenously express adenylyl cyclase isoforms I, III, V, VI, VII and IX, which are divided into four groups based on their selectivity for G proteins (Table 1.1, Tang *et al.* 1992, Sabbatini *et al.* 2016). With that in mind, I decided to overexpress one adenylyl cyclase isoform from each group in the presence or absence of GPSM3. For Group I, I studied adenylyl cyclase III, which is stimulated by $G\alpha_s$ and forskolin but inhibited by $G\alpha_i$ and Gβγ. In the presence of GPSM3, there was a 34.8% increase in maximal DMB-forskolin activity when compared to control ($p < 0.05$). This is consistent with the ability of GPSM3 to inhibit $G\alpha_i$-mediated signalling, whereas it would not appear to reflect any GPSM3-induced enhancement of Gβγ-mediated inhibition.

Next, I investigated the effect of GPSM3 on adenylyl cyclase VI from Group II, which is insensitive to $G\alpha_i$ and stimulated by $G\alpha_s$, Gβγ and forskolin. Results indicate that presence of GPSM3 has no effect on the forskolin concentration dependence curve when compared to control. Again, this neutral finding suggests that the stimulatory effect of Gβγ is unaltered by GPSM3 while any effect on $G\alpha_i$ signalling would be without consequence on the activity of this adenylyl cyclase subtype.

Finally, to examine the effect on Group III adenylyl cyclases, I overexpressed isoform IV, which is naturally inhibited by $G\alpha_i$ and stimulated by $G\alpha_s$, $G\beta\gamma$ and forskolin. Co-tranfection with GPSM3 increased efficacy by 21.6% when compared to control ($p < 0.05$). Similar to observations with adenylyl cyclase subtype III, this is indicative of a GPSM3-induced decrease in $G\alpha_i$-mediated signalling with no appreciable effect on $G\beta\gamma$. Taken together, these results suggest that GPSM3 works on different isoforms of adenylyl cyclases via an inhibitory effect on $G\alpha_i$-mediated signalling. Canonically, GPSM3 promotes $G\beta\gamma$-mediated signalling; however, there is no clear effect of such signalling within these adenylyl cyclase-based experiments.

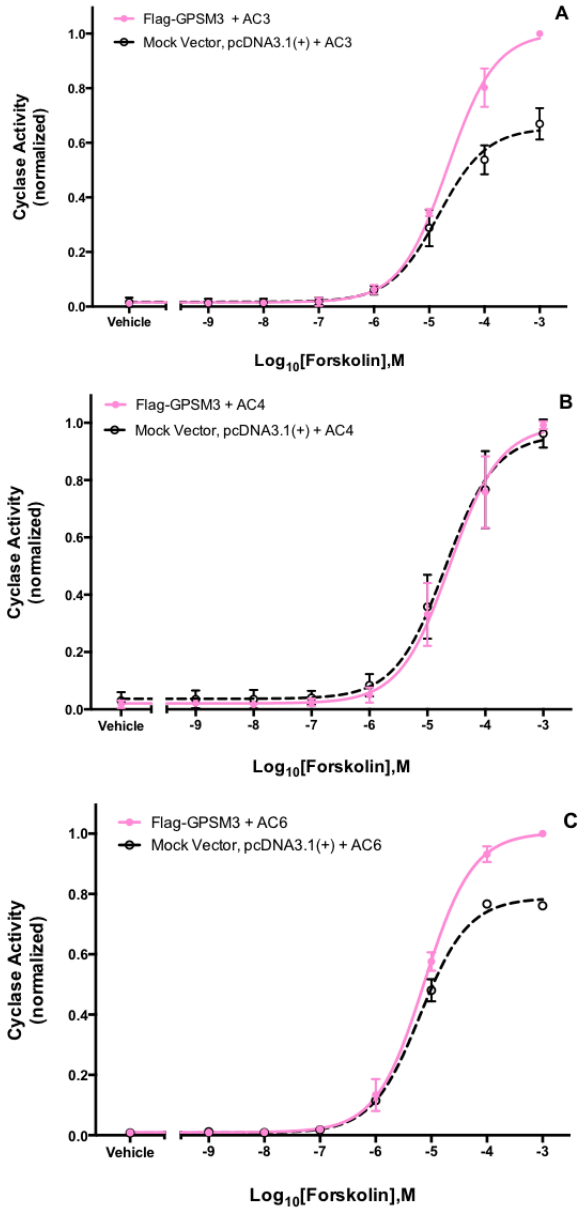

Fig. 3-10. GPSM3 increases the efficacy of DMB-forskolin on adenylyl cyclases III and VI with no effect on IV. HEK293H cells were co-transfected with GloSensor™ cAMP biosensor and one of three different isoforms of adenylyl cyclase (III, IV, VI), in the presence or absence of GPSM3 as described in *Materials and Methods*. Cells were treated with IBMX (400 μM) for 20 mins and then stimulated with DMB-Forskolin (1 nM – 1 mM) or vehicle. Data were normalized as a fraction of the greatest value of cyclase activity in each experiment. Values shown are means

± SEM (n = 3 independent experiments, each performed in triplicate). (**A**) GPSM3 significantly increased potency and efficacy of exogenous ACIII when compared with control. $LogEC_{50}$ values for Flag-GPSM3 and control were -4.67 ± 0.03 and -4.83 ± 0.06, respectively; differences were statistically significant ($p < 0.05$). Maximal fitted values indicative of efficacy for data acquired in the presence and absence of Flag-GPSM3 were 1.00 ± 0.01 and 0.65 ± 0.01, respectively; differences were statistically significant ($p < 0.0001$). (**B**) GPSM3 has no effect on fitted $LogEC_{50}$, upper and lower asymptotic values of ACIV (p = 0.5282, 0.5148, 0.4820, respectively). (**C**) GPSM3 significantly increased the maximal effect of DMB-forskolin on exogenous ACVI. $LogEC_{50}$ values for DMB-forskolin acquired in the presence and absence of Flag-GPSM3 were -5.13 ± 0.02 and -5.20 ± 0.03, respectively; differences were not statistically significant (p = 0.0821). Maximal fitted values for DMB-forskolin in the presence and absence of Flag-GPSM3 were 1.00 ± 0.01 and 0.787 ± 0.009, respectively; differences were statistically significant ($p <$ 0.0001). All statistical values were based on extra sum of squares F-tests.

Chapter 4

4 DISCUSSION

4.1 SUMMARY OF FINDINGS

In recent years, novel accessory proteins for GPCRs and G proteins have been discovered (Cismowski, 2006, Sato *et al.* 2006); however, their abilities to modulate receptor signalling have yet to be fully understood. Thus, studies in this thesis have focused on one particular accessory protein called GPSM3 (also known as AGS4 or G18). The overall objective of this research was to evaluate how GPSM3 could regulate GPCR signalling. The specific aims of this research were as follows:

1. To characterize the effects of GPSM3 on β-arrestin recruitment to $G\alpha_s$, $G\alpha_I$ and $G\alpha_{q/11}$-coupled receptors after agonist stimulation
2. To evaluate which protein domains within GPSM3 are important for GPCR signalling
3. To determine the effect of GPSM3 on GPCR-regulated adenylyl cyclase activity

The studies presented herein reveal valuable aspects of how a GPSM domain-containing protein may influence GPCR signalling within a cellular context. Studying how G protein activation states may be altered through nucleotide exchange by cytosolic proteins not only allows us to further understand G protein regulatory potential but also helps us get a better idea of how GPCR signalling may be influenced under normal and pathophysiological environments.

The present studies extend the role of GPSM3 as a protein capable of selectively modulating $G_{i/o}$-coupled GPCR signalling. Specifically, the effect of GPSM3 on β-arrestin recruitment to $G_{i/o}$-coupled receptors manifests as a decrease in signal amplitude, which corresponds to a decrease in β-arrestin-receptor interaction, with no observable change in potency. The selective reduction in β-arrestin recruitment to $G_{i/o}$-coupled receptors suggests that GPSM3 could: i) directly influence β-arrestin recruitment by occluding the binding site on the receptor and/or ii) indirectly decrease recruitment by interfering with downstream signalling molecules, resulting in decreased β-arrestin localization to the receptor. The data presented in this thesis show that GPSM3 not only decreases agonist-stimulated β-arrestin recruitment but also decreases basal signal. This suggests that GPSM3 can exert its effects without the presence of an agonist, which is consistent with its ability to bind to $G\alpha_i$ proteins independently of receptor activation.

GPSM3 failed to exert any measurable effect on β-arrestin recruitment to G_s- or $G_{q/11}$-coupled GPCRs. This implies that the mechanism by which GPSM3 acts is specific towards $G\alpha_i$ proteins and their associated receptors. The key difference between G_i-, G_s- and $G_{q/11}$-receptor signalling complexes is the Gα protein subtypes (*i.e* $G\alpha_{i/o}$ *vs* $G\alpha_s$ *vs* $G\alpha_{q/11}$) they couple to. It is possible that differences in tertiary structure and amino acid sequence can in part, impact the degree of binding of GPSM3 to these G proteins, which could then influence the ability of GPSM3 to carry out its function as a GDI (Kimple *et al.* 2004, Giguere *et al.* 2012). The characterized binding interactions between GPSM3 and GDP-$G\alpha_i$ and the lack of binding to other G protein subfamilies, may in part reflect why GPSM3 was found to solely affect G_i-coupled signalling (Giguere *et al.* 2012, Willard *et al.* 2008). In addition, it is possible that the

GPSM3/GDP-Gα_i interaction could promote G$\beta\gamma$-mediated signalling, thereby depleting or re-routing the following processes: i) phosphorylation of the C-terminal portion of the receptor by GRK, ii) β-arrestin recruitment to the phosphorylated receptor and/or iii) interfering with other proteins that contribute to receptor internalization, such as clathrins and other adaptor proteins.

My findings suggest that both the N-terminus and GPSM motifs of GPSM3 contribute to reducing the interaction between β-arrestin and receptors in both the presence and absence of agonist. It appears that the arginine to phenylalanine amino acid substitution in part of the conserved Asp/Glu-Gln-Arg triad within each GPSM motif reduces the affinity between GPSM3 and GDP-Gα_i and it is conceivable that such mutations may modify the tertiary structure in a way that disrupts the stability of GPSM3, thus decreasing the amount of available protein able to bind to Gα_i proteins. Additionally, reduced interactions due to these mutations could promote the reconstitution of the G$\alpha\bullet\beta\gamma$ heterotrimeric complex, thereby altering G protein-mediated signalling by either promoting Gα_i or impeding G$\beta\gamma$. Physiologically, this phenomenon may contribute to the differences in susceptibility to autoimmune diseases seen in a subset of single-nucleotide polymorphisms of GPSM3, as reported in Sirota *et al.* 2009 and Corona *et al.* 2010. Compelling genome-wide association studies have discovered protective single nucleotide polymorphisms (rs204989 and rs204991) on allele C within the locus gene of GPSM3 to be less prevalent among autoimmune disease patients (Gall *et al.* 2016). These alleles have been associated with a reduced incidence of rheumatoid arthritis, which could be attributable to altered GPCR signalling within cells involved in this disease (Sirota *et al.* 2009, Corona *et al.* 2010). GPSM3 has been positively associated with the migration of cells involved in inflammation, therefore it is possible that these SNPs protect individuals from rheumatoid arthritis by reducing GPSM3 function and thus, decreasing inflammatory cell migration and subsequent infiltration to

sites of action (*e.g.* joints). Moreover, the N-terminal domain could also contribute to the effects seen by facilitating binding between $G\alpha_i$ and GPSM3 but also interactions within the protein itself. Thus, besides the three GPSM motifs, GPSM3 contains an additional Gα protein binding domain on the N-terminus for both $G\alpha_i$ and $G\alpha_o$ subtypes, which could also be important for its regulation in GPCR signalling. Research in our lab shows that the N-terminal domain acts as a GEF for $G\alpha_{i1}$, while conversely acting as a weak GDI on $G\alpha_o$ (Zhao *et al.* 2010). This supports the finding in Figure 3-3 that both N-terminus and GPSM motifs may be necessary to facilitate appropriate binding interactions with $G\alpha_{i/o}$ proteins to carry out its function in GPCR signalling. Future studies using co-immunoprecipitation may help to elucidate whether the reduction of β-arrestin recruitment as seen with GPSM3 is attributable to its binding interactions with $G\alpha_{i/o}$.

The prevailing view of the β-arrestin-dependent pathway has been that signal termination is largely G protein-independent (Rajogopal *et al.* 2010, Shenoy *et al.* 2016, Grundmann *et al.* 2018). Although the aforementioned results do not directly indicate how GPSM3 may be decreasing the ability of β-arrestin to bind to G_i-coupled receptors, a study by Grundmann *et al.* 2018 suggests that the presence of active G proteins may be crucial for arrestin-dependent signalling. In these experiments, $G\alpha_s$ and $G\alpha_{q/12}$ proteins were knocked out in HEK293 cells using CRIPSPR/Cas9 technology, while the $G\alpha_{i/o}$ family was inactivated using pertussis toxin. Cells lacking functional G proteins exhibited a large reduction in ERK1/2 phosphorylation, a well-known β-arrestin-dependent signalling pathway. Notwithstanding the fact that ERK1/2 cascade is well downstream of the GPCR and G protein as well as the fact that such signalling could occur possibly without the need for Gα proteins, it is possible that G protein activation may still be necessary for receptor signalling termination. This idea has been supported by the

observation in this thesis that overexpression of $G\beta_1\gamma_2$ mimics the effects of GPSM3 by reducing the interaction between β-arrestin-1 and G_i-coupled GPCRs, specifically α_{2A}-adrenergic and μ-opioid receptors (Figure 3-5). Moreover, my data illustrating that co-transfection with a GRK2ct, a Gβγ inhibitor, rescues the inhibition of β-arrestin-1 recruitment by GPSM3 further supports the idea that G protein activation, specifically the liberation of Gβγ, may be important in β-arrestin-mediated receptor desensitization (Figure 3-6).

In the absence of $G\alpha_i$ activation due to treatment with pertussis toxin, GPSM3 was still capable of reducing the GPCR/β-arrestin binding interaction. This observation is consistent with the fact that GPSM3 can bind to $G\alpha_i$ independently of receptor activation and foster the sequence of events leading to a reduction in β-arrestin binding. Moreover, this interaction could promote the dissociation and subsequent release of Gβγ, supporting the idea that nucleotide exchange may not be required for G protein activation, specifically in the case for Gβγ. Precedent studies argue that Gβγ can cultivate GRK mobilization towards the plasma membrane and thereby, increase the rate of receptor phosphorylation and subsequent binding of arrestins (Touhara *et al.* 1994, Boekhoff *et al.* 1994); however, my findings as reported here suggest that the real situation at the cellular level may be more complicated than originally thought. Increased levels of $G\beta_1\gamma_2$ seem to mimic the effect of GPSM3, which falls in line with the idea that Gβγ activity is promoted directly via GPSM3/$G\alpha_i$ binding interaction. What is perhaps even more interesting is that inhibiting Gβγ activity with a short Gβγ-binding peptide appears to restore β-arrestin recruitment to G_i-coupled receptors in the presence of GPSM3. This strongly suggests that Gβγ activation may be necessary for β-arrestin recruitment under certain conditions. Thus, it is possible that GPSM3 could function by altering stoichiometric ratios of free G proteins,

specifically Gβγ, which in turn can modulate receptor desensitization pathways. This could be tested by carrying out a series of co-immunoprecipitation assays with subsequent immunoblotting to measure the amount of Gβγ in cells co-transfected with GPSM3 or control vector. In addition, another way to test this Gβγ-mediated effect is by overexpressing Gβγ or control vector in a G_s or $G_{q/11}$-coupled GPCR system and examine the differences in interactions between β-arrestin and receptor. My prediction is that Gβγ will have an effect similar to that seen in G_i-coupled GPCRs as shown in Figure 1-1. The rationale for this expectation is that in a G_i-coupled GPCR system, GPSM3 can free up Gβγ by binding to $G\alpha_{i/o}$ and displacing Gβγ, but can also prevent Gβγ re-association back to $G\alpha_{i/o}$ by occluding the Gβγ-binding site. This is not seen in my results for G_s or $G_{q/11}$-coupled receptors, perhaps because GPSM3 cannot bind to the Gα subtypes and therefore release free Gβγ through the aforementioned mechanism.

In contrast to the present findings which suggest that GPSM3 competes with Gβγ for $G\alpha_{i/o}$, a study by Giguere *et al.* 2012 has reported that GPSM3 can bind directly to all four Gβ subunits via a leucine-rich region proximal to the N-terminus of the first GPSM motif, thereby storing a potential to decrease Gβγ-mediated signalling; however, this has not been reproduced by other labs, including ours (Zhao *et al.* 2010, Wallace, D, 2017, MSc thesis, University of Western Ontario). Although the role of Gβγ in these studies is confusing, the idea that GPSM3 could act as chaperone for Gβ is not unreasonable. In addition, while it could be true that GPSM3 binds to both $G\alpha_{i/o}$ and Gβγ subunits, it would be necessary to know whether it forms a quaternary complex with all three subunits or whether it binds to either $G\alpha_{i/o}$ or Gβγ at any given time. To do so, it would be necessary to carry out experiments solving for the molecular structure of GPSM3 complexed with the heterotrimeric G protein.

Although the present findings do not directly speak to how exactly GPSM3 produces its effects within the cell, my observations are consistent with the idea that the complex formed by GPSM3 and GDP-$G\alpha_i$ could be potentially occluding the serine and threonine residues on C-terminal regions of the receptor (Oner *et al.* 2010b). This occlusion could then prevent the ability of GRK to phosphorylate those residues, which would inevitably lead to a decrease in phosphorylation (Figure 4-1). This in turn, could subsequently decrease the rate and affinity of β-arrestin recruitment, which would fall in line with our observations. However, this theoretical mechanism seems very unlikely given that overexpression with a $G\beta_1\gamma_2$ inhibitor reverses the effect of GPSM3, which seems to point to a mechanism associated with increased free Gβγ at or near the receptor due to the presence of GPSM3. Furthermore, the GPSM3-GDP-$G\alpha_i$ complex could also block interactions between the receptor and other proteins (*e.g.* β-arrestins) by directly coupling to the receptor, perhaps even after receptor phosphorylation (Oner *et al.* 2010b).

Some possible interpretations of the present findings are suggested by functional studies on Gβγ and its binding interactions with proteins involved in signal termination, specifically GRKs and β-arrestins. There is evidence that Gβγ plays an integral role in bringing both GRKs and β-arrestins to the receptor, shortly after receptor activation (Boekhoff *et al.* 1994, Ferguson *et al.* 1996); however, increasing the amount of free Gβγ via GPSM3 could disrupt the balance in GPCR signalling, ergo GRK or β-arrestin binding could be decreased. Therefore, Gβγ dimer could alter signalling by: i) directly binding to a subset of Gβγ-sensitive GRKs (mainly GRK2 and GRK3) and preventing their localization to the C-terminal portions of receptors (Figure 4-2), ii) directly binding to β-arrestins and slowing down the rate of recruitment to phosphorylated residues on the receptor (Figure 4-3) and/or iii) indirectly activating/repressing downstream

effectors involved in signal termination. One way to distinguish between these possibilities would be via the evaluation of the effects of GPSM3 on receptor phosphorylation states. This can be achieved for example via co-immunoprecipitation and/or immunoblotting experiments using receptor-specific phospho-antibodies. A decrease in phosphorylation would be consistent with a decrease in GRK2/3 function due to liberation of Gβγ by GPSM3 to a position that does not facilitate productive GRK/GPCR contact. Conversely, an increase in phosphorylation partnered with a decrease in β-arrestin-receptor interaction would be indicative of a problem on the level of β-arrestin recruitment. This could also be in part ascribed to the increase in free Gβγ by GPSM3, which could directly alter β-arrestin activity. Despite enormous advances in our understanding of G protein- and arrestin-mediated processes, their interplay with one another remains incompletely understood. This could be in part accredited to the lack of sufficient tools to measure connections between the two proteins.

I also examined the potential influence of GPSM3 on adenylyl cyclase activity and the inhibition thereof by $G_{i/o}$-coupled receptors. Given the biochemical ability of GPSM3 to bind to and inhibit nucleotide exchange on $G\alpha_i$, I initially decided to examine its effects on the $G_{i/o}$-coupled adenosine A1, α_{2A}-adrenergic and μ-opioid receptors, via changes in cAMP production. I predicted that GPSM3 would reduce the degree and/or agonist potency of $G\alpha_i$-mediated inhibition on forskolin-stimulated adenylyl cyclase activity; however, in these sets of experiments, I observed that GPSM3 significantly reduced basal cAMP production. In addition, there was a slight increase in the percentage inhibition of adenylyl cyclase activity due to the activation of μ-opioid receptor in the presence of GPSM3 when compared to control (Figure 3-7). This suggests that GPSM3 is increasing inhibition on adenylyl cyclase activity.

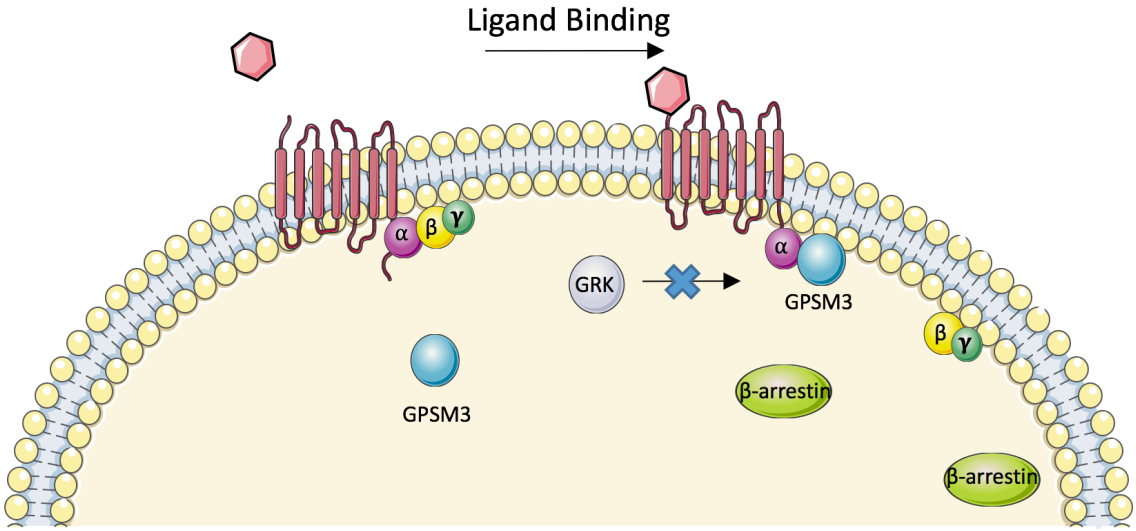

Figure 4-1 Presence of GPSM3 may decrease receptor phosphorylation by blocking activity of GRKs. The complex formed by GPSM3 and $G\alpha_{i}$-GDP could potentially occlude the serine and threonine residues on C-terminal region of GPCR, which would sterically block the ability of G protein receptor kinases (GRKs) to phosphorylate those sites. Decreased phosphorylation by GRKs will lead to an inevitable decrease in β-arrestin recruitment, thereby reducing the amount of receptor being removed from the plasma membrane.

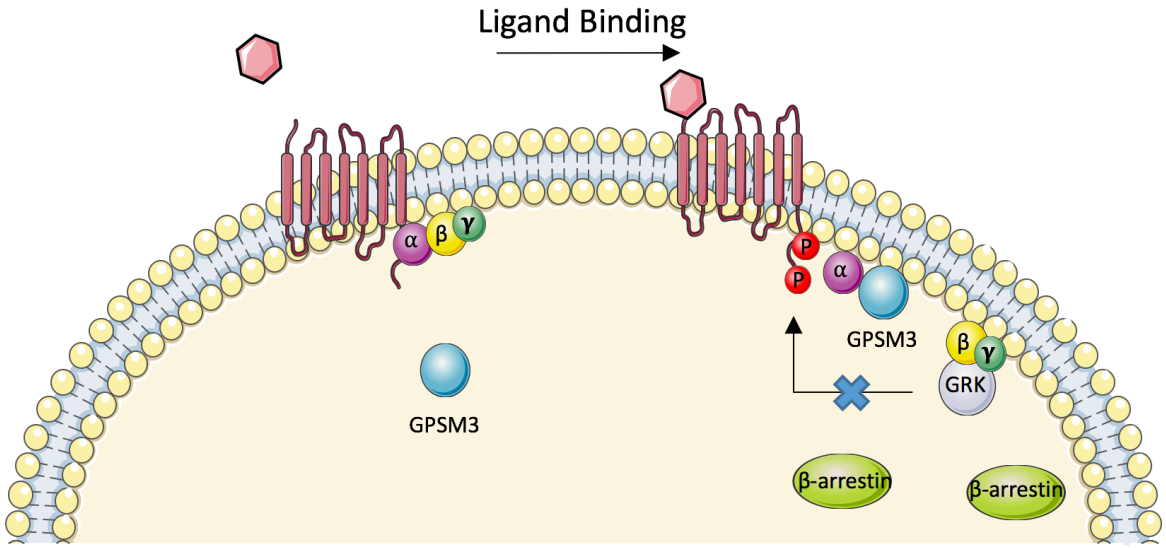

Figure 4-2 Liberation of Gβγ by GPSM3 may decrease GRK2/3 activity. When GPSM3 binds to GDP-bound forms of $G\alpha_i$ proteins, the interaction may promote the dissociation of the Gβγ dimer and at the same time may delay its re-association to the heterotrimeric G protein complex. Gβγ can therefore bind to and slow down recruitment of GRK proteins to the C-terminal region of GPCR, thus preventing receptor phosphorylation.

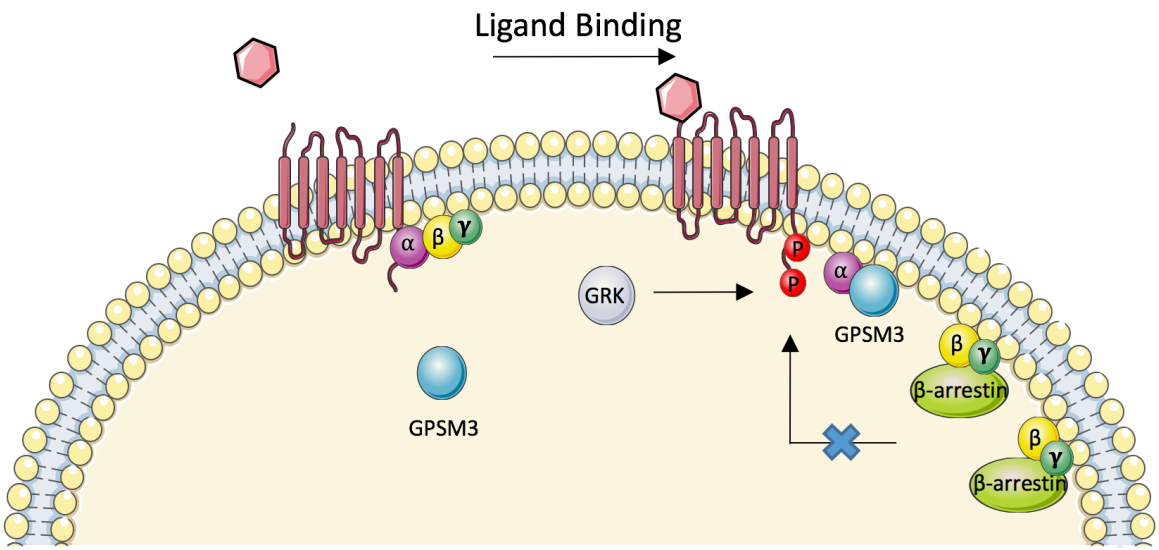

Figure 4-3 GPSM3 decreases β-arrestin recruitment to $G_{i/o}$-coupled receptors via a Gβγ-dependent mechanism. The complex formed by GPSM3 and GDP-bound $G\alpha_i$ facilitates Gβγ-mediated signalling. The Gβγ dimer could alter signalling by binding to and inhibiting recruitment of β-arrestin to the phosphorylated C-terminal region of GPCR. As a consequence, these interactions prevent receptor desensitization and other β-arrestin signalling pathways (not shown above).

Since GPSM3 is inhibiting $G\alpha_I$ protein activity, it seems unlikely that this increase in inhibition is coming from this G protein. GPSM3 also promotes the liberation of free $G\beta\gamma$, which is known to have inhibitory potential on certain isoforms of adenylyl cyclase, so it is possible that this increase in inhibition could be a direct result of this (Sunahara *et al.* 2002) On the other hand, GPSM3 had no apparent effect on adenylyl cyclase activity when cells were transfected with the G_s-coupled β_2-adrenergic and parathyroid hormone receptors. This lack of effect is consistent with the ability of GPSM3 to selectively bind to and alter $G\alpha_i$ proteins. In addition, the ability of GPSM3 to reduce DMB-forskolin-stimulated cAMP production in the absence of any agonist reinforces the notion that GPSM3 can mediate its effects independently of receptor activation. The ability of GPSM3 to reduce basal DMB-forkolin-stimulated (but not G_s-stimulated) adenylyl cyclase activity is consistent with the observed ability of GPSM3 to decrease DMB-forskolin potency on endogenous adenylyl cyclase activity (Figure 3-9); however, the underlying mechanism for these observations is not obvious. One likely reason behind these findings is modulation of G protein activity. We know that GPSM3 can bind to and impede $G\alpha_i$-mediated signalling but this effect was not present in our results, as shown by inhibition of adenylyl cyclase (Figure 3-7). GPSM3 can promote $G\beta\gamma$ release, which can in turn directly activate or inhibit different isoforms of adenylyl cyclase activity (Sunahara *et al.* 2002, Table 1.1). Thus, it is possible that $G\beta\gamma$ could have a net inhibitory, stimulatory or neutral effect on cAMP production, depending upon the type and expression of adenylyl cyclase isoforms present. HEK-293 cells endogenously express adenylyl cyclase isoforms I, III, V, VI, VII and IX at varying levels (Atwood *et al.* 2011). Based on the reported sensitivities of these isoforms (Table 1.1), it is possible that this particular combination of endogenous adenylyl cyclases would exhibit a net inhibitory response to GPSM3-promoted $G\beta\gamma$ activity. Therefore, in order to further understand

how GPSM3 might be mediating its observed inhibitory effect (as implied by the observed decrease in DMB-forskolin activity), it might be helpful to know the relative proportions of these isoforms and be able to overexpress them in the presence and absence of GPSM3.

My initial prediction was that GPSM3 would inhibit adenylyl cyclase III activity by promoting Gβγ release, which has an inhibitory effect (Sunahara *et al.* 2002, Table 1.1). Contrary to this prediction, as compared to the mock vector control, the overexpression of GPSM3 together with adenylyl cyclase III increased the maximal effect of DMB-forskolin. This observation is not consistent with increased Gβγ effects, but rather suggests that GPSM3 under these conditions serves to increase stimulation by DMB-forskolin by reducing the inhibitory effect of $G\alpha_i$. When adenylyl cyclase IV was overexpressed, I expected to see an increase in adenylyl cyclase activity when cells were co-transfected with GPSM3. This isoform is insensitive to $G\alpha_i$ and stimulated by Gβγ. My prediction was that since GPSM3 inhibits $G\alpha_{i/o}$ while promoting Gβγ, the next effect would be stimulation (Sunahara *et al,* 2002, Table 1.1). In contrast to these expectations, GPSM3 had no measureable effect on adenylyl cyclase IV activity when compared to control. Again, this observation is not consistent with increased Gβγ effects, but is consistent with the interpretation that GPSM3 may have inhibited $G\alpha_{i/o}$ under these conditions, an effect to which adenylyl cyclase IV would presumably be insensitive. Lastly, I decided to overexpress adenylyl cyclase VI, which is inhibited by $G\alpha_{i/o}$ and stimulated by Gβγ. Our initial prediction was that the presence of GPSM3 would increase adenylyl cyclase VI activity by inhibiting inhibition by $G\alpha_{i/o,}$ while simultaneously increasing stimulation by promoting Gβγ release. Yet again, the observed result is not consistent with increased Gβγ effects, but rather suggests that GPSM3 under these conditions serves to increase stimulation by

DMB-forskolin by reducing the inhibitory effect of $G\alpha_{i/o}$ on adenylyl cyclase VI activity. Taken together, the simplest explanation for these observed effects of GPSM3 on the various adenylyl cyclase subtypes tested is that the activation by GPSM3 of endogenous Gβγ is not sufficient to produce measurable effects on the enzymes when overexpressed, whereas inhibition of endogenous $G\alpha_{i/o}$ subunits by GPSM3 is sufficient to measurably obstruct their basal inhibitory effect on ectopically expressed adenylyl cyclases III and VI. Moreover, it could be difficult to observe endogenous Gβγ protein effects on exogenously expressed adenylyl cyclases, especially after stimulation with a powerful cyclase specific agonist (*e.g.* forskolin). As well, it is possible that altering the stoichiometric ratios between G proteins and effectors by overexpressing the latter but not the former may obscure some of their biological interactions. While it is possible that Gβγ may stimulate or inhibit these isoforms, perhaps endogenous levels of these G proteins are not sufficient to facilitate the adequate amount of activity on overexpressed adenylyl cyclases. Again, my findings fall in line with the idea that GPSM3 under such conditions is working to inhibit $G\alpha_{i/o}$-signalling, whereas its effects on Gβγ are unclear.

4.2 Contributions of research to current state of knowledge

There is still some ambiguity regarding whether or not GPCR signal termination is dependent on G protein activity (Rajogopal *et al.* 2010, Shenoy *et al.* 2016, Grundmann *et al.* 2018). The classical paradigm purports that receptor phosphorylation and subsequent β-arrestin binding is independent of G proteins (Wei *et al.* 2003, Van Koppen *et al.* 2004); however, novel research suggests that “zero functional G” cells or cells with collectively eliminated or inactive G proteins exhibit diminished β-arrestin signalling, specifically phosphorylation of ERK1/2 in the

MAP kinase pathway (Grundmann *et al.* 2018). This finding introduces the idea that activation of G proteins may indeed be, in part, necessary for processes involved in receptor desensitization. The observations by Grundmann *et al.* 2018 are both complemented and contrasted by our findings, which largely suggest that G proteins (specifically Gβγ) do in fact play a role in regulating the rate of β-arrestin recruitment. The study by Grundmann and co-workers shows that complete knockout of $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ subtypes via CRISPR/Cas9 technology simultaneously with a "knock down" of $G\alpha_{i/o}$ using PTX is detrimental to the recruitment of β-arrestin. In direct contrast to this, my results suggest that activation of $G\alpha_{i/o}$ is not necessarily required for recruitment, and that activation of Gβγ under certain circumstances can reduce GPCR-β-arrestin binding. A potential shortcoming in their experimental design, which could account for the differences seen between results, is that they did not measure G protein activity at a proximal step (*e.g.* GTPase or cAMP measurement in the presence and absence of PTX) and they also did not test out whether $G\alpha_{i/o}$ activation is necessary for β-arrestin recruitment, independently from Gβγ activation or vice versa. Furthermore, they did not knock out endogenous Gβγ, which could also be a contributing factor to the differences seen. Thus, their interpretations omit the influence of individual G proteins on β-arrestin recruitment in HEK293 cells. Even though the role of G proteins in this pathway is still unclear, these findings pave an avenue for future discoveries and could potentially be profitable within the pharmaceutical world.

Knowledge surrounding accessory proteins, such as RGS proteins and GRKs, has accumulated for more than two decades; however, information on AGS proteins, specifically GPSM3, has been limited. Even though an appreciable number of research articles have been

published on GPSM3 since its discovery in 2004, there is still some uncertainty as to how this protein functions within the cell (Cismowski *et al.* 2006, Oner *et al.* 2010, Giguere *et al.* 2012, Giguere *et al.* 2013). Studies have shown that cells lacking GPSM3 exhibit lower rates of monocyte and leukocyte migration to chemotactic agents (*e.g.* CCL19 and N-Formylmethionine-leucyl-phenylalanine) and that expression is linked to inflammatory pathways (Giguere *et al.* 2013, Sunderkotter *et al.* 2004); however, the mechanism underlying these events occur is still under investigation. There is some evidence that the Gβγ-mediated pathway may be implicated in cell migration and if this were true, it would suggest a mechanism as to how GPSM3 may be altering GPCR signalling to promote cell migration. One supposition may be that GPSM3 is working by virtue of increasing free Gβγ and thus, stimulating pathways associated with cell migration. Another possible interpretation may be that GPSM3 is decreasing inhibition of $G\alpha_{i/o}$-mediated signalling and thereby, relieving any inhibitory pathways that decrease cell mobility. Furthermore, our results suggest that GPSM3 decreases β-arrestin recruitment to G_i-coupled GPCRs, which could include chemokine receptors. In theory, such decrease would prolong the longevity of the receptor at the plasma membrane and therefore prolong chemokine signalling, which involves cell migration. Lastly, there is some evidence of GPSM-containing proteins co-localizing to the nucleus, which suggests that GPSM3 could potentially act as a transcription factor that regulates the production of mRNA genes involved in cell migration (Giguere *et al.* 2012).

My observations contribute to the current state of knowledge by shedding new light on GPSM3 function. My findings imply that GPSM3 can inhibit $G\alpha_{i/o}$-mediated signalling through adenylyl cyclase activity. Furthermore, GPSM3 as shown here can indirectly inhibit β-arrestin recruitment to G_i-coupled receptors by promoting release and activity of Gβγ. Although the

relationship between Gβγ and cell migration is unclear, the ability of GPSM3 to promote Gβγ and be associated with cell migration would be an interesting avenue for exploration. In addition, my results strongly suggest that GPSM3 can affect $G\alpha_I$ protein activity independently of receptor activation and alter crucial stoichiometric ratios of free G proteins which could in turn alter the signalling cascades involved in receptor desensitization.

The ability of GPSM3 to decrease β-arrestin recruitment specifically to G_i-coupled receptors could be beneficial in promoting the lifetime of receptor signalling at the plasma membrane. For example, the evidence for GPSM3 in reducing μ-opioid receptor desensitization would, in theory, promote μ-opioid-related analgesic effects. If this were to be true, this would be a useful tool for pharmaceutical companies in generating a drug capable of mimicking the GPSM3 effect to promote these events. There is evidence that β-arrestin activity is increased after treatment with μ-opioid agonists, which can lead to undesirable symptoms often seen with opioid-use, such as gastrointestinal dysfunction (*i.e.* constipation) and respiratory depression (Madariaga-Mazon *et al.* 2017). My data suggests that GPSM3 may potentially carry out its function by binding directly to β-arrestins, which could then decrease any β-arrestin-mediated downstream signalling and side effects, such as the symptoms associated with opioid-use. Furthermore, the data presented in this thesis suggest that GPSM3 could function by altering the stoichiometry between free G proteins (*i.e.* Gα and Gβγ), β-arrestins and/or GRKs, which could influence their associated signalling pathways. This strongly suggests that changes in protein stoichiometry may drastically alter the function and sequence of events. The study by Pitcher and colleagues suggests that stoichiometric ratios between Gβγ and GRK proteins are fundamental in initiating optimal rates for receptor desensitization (Pitcher *et al.* 1992). This is consistent with

my results, which imply that disrupting this balance by overexpressing Gβγ could impede the maximal rate for β-arrestin recruitment to the receptor (Figure 3-5). Although the importance of these ratios is still to be determined, it would be wise to take these stoichiometric ratios into more consideration when evaluating the sequelae of GPCR-mediated signalling pathways.

4.3 Limitations of study

Data obtained in this thesis primarily utilized a live cell GloSensor^{TM} assay for direct measurement of cAMP levels and a luminescence-based protein complementation assay for β-arrestin recruitment. Collectively, these two assays both studied changes in bioluminescence over time. One evident limitation to this is that the results observed were not repeated using an alternative method in order to determine whether or not results seen were due to an artifact (*i.e* direct measurement of cAMP, co-immunoprecipitation, BRET, FRET); however, this is very unlikely given that I used appropriate positive and negative controls. Furthermore, it is unclear whether the protein complementation assay used is reversible. With several of the GPCRs tested, I found that bioluminescence, specifically in the case for the β-arrestin assay, continued to rise well beyond the given experimental timeline (*e.g.* 80 mins). Reversibility of β-arrestin-receptor binding could be experimentally tested by treating cells with a receptor-specific antagonist shortly after agonist stimulation. If cells show decreased luminescence in response to antagonist treatment and then are again able to produce luminescence after subsequent stimulation with dose-dependent concentrations of agonist, this would demonstrate that the complementation assay is indeed reversible.

As with other cell-based studies, transfection efficiencies could potentially vary between different experiments. My transfection efficiency in HEK-293H cells usually ranged from 70-80% as discussed in *Materials and Methods.* Likewise, the expression levels of transfected protein constructs could conceivably vary between conditions and within particular experiments, given the observed GPSM3-related changes in GPCR expression in some (but not all) studies (Giguere *et al.* 2013, Easton *et al.* 2007, Barberis *et al.* 2008); however, this seems unlikely considering the consistency between the results shown in Figures 3-2, 3-6 and 3-8. Moreover, the cytotoxic nature of the transfection reagent X-tremeGene and overexpression of various plasmids does not reflect what would happen under physiological conditions. Furthermore, not only could plasmids be expressed in different proportions, they could also produce a byproduct which could interfere with the natural cellular machinery. Lastly, there could be a relative excess of one or the other luciferase fragments in complementation-based experiments, which could also affect agonist concentration dependence.

Due to time constraints, the μ-opioid receptor construct that was used in the cyclase assays was the same construct used in the β-arrestin assay, which contained a fusion-protein (modified to include a complementary luciferase domain) at the C-terminal portion of the receptor. This tag could potentially interfere with G protein activation and therefore, the extent of adenylyl cyclase activity. Another limitation is that only one cell type was used and results found thus far cannot be generalized to other cells types. Furthermore, HEK-293H cells naturally lack GPSM3, so by transfecting this protein into this cell type we are creating an environment that is not naturally found under physiological conditions.

It would be useful to examine whether the observations made here extend to *in vivo* situations. However, after many research attempts, I was unable to produce GPSM3 knockout mice, thus the focus of this research had to change from studying the role of GPSM3 in diabetic nephropathy to identifying the function of GPSM3 in GPCR signalling (Figure A-1, Figure A-3). Therefore, it is very difficult to translate effects seen *in vitro* to *in vivo* models. Furthermore, it is possible that certain drugs may have multiple target sites along the cell membrane. This could be a potential confounding variable when drugs have off target effects on different receptors. These off-target effects could alter the results seen within the cell and could be mistakenly attributed to GPSM3. For example, in one of the β-arrestin assays conducted, I stimulated cells transfected with muscarinic type-1 receptor with the agonist carbachol. Carbachol can also stimulate nicotinic receptors, also found in the plasma membrane of cells, including HEK-293H (Boksa *et al.* 1987). Despite these off-target effects seen under normal conditions, the signal received from that assay is solely attributable to the interaction between β-arrestin-1 and muscarinic type-1 receptor because only the transfected constructs would contain the fusion-proteins (modified to include a complementary luciferase domain) capable of restoring the functional enzyme responsible for catalyzing the D-luciferin substrate into the bioluminescent oxy-luciferin. Therefore, these off-target drug effects would be more important to consider in the cAMP assays conducted. Furthermore, there is evidence that GPSM3 has multiple binding partners within the cell, such as 14-3-3 and RGS5, which on their own could affect GPCR signalling. In addition, it is possible that GPSM3 may be directly interacting with β-arrestin proteins, which on their own modulate various aspects of signalling, such as MAP kinase and Src activation (Lefkowitz *et al.* 2006). Therefore, GPSM3 could alter activities of these intracellular proteins which could possibly produce cellular changes observed within these experiments.

4.4 Future directions

In future studies, the effects of GPSM3 on G_i–coupled signalling ought to be further characterized *in vitro* to define the GPSM3-dependent mechanisms that reduce the magnitude of β-arrestin recruitment. Thus, comprehensive expression and protein analysis of GPSM3 and additional binding interactors should be investigated. In order to further elucidate the mechanism underlying the ability of GPSM3 to reduce β-arrestin recruitment to G_i-coupled receptors, it is imperative that we study the phosphorylation state of the receptors. This should give us a better understanding of whether or not the observed decrease in β-arrestin recruitment to $G_{i/o}$-coupled receptors is due to a decrease in phosphorylation by GRK or due to a decrease in β-arrestin binding. Another way to study whether phosphorylation is an issue is by studying the effects of Gβγ-sensitive and insensitive GRK isoforms. If GPSM3 is producing its inhibitory effects on GPCR-β-arrestin binding via a Gβγ-dependent mechanism wherein preventing recruitment of GRK2/3 results in a lack of receptor phosphorylation, then overexpressing a Gβγ-insensitive GRK, such as GRK4, ought to rescue the effect seen with GPSM3. If the GPSM3 effect persists, this would instead suggest that perhaps phosphorylation is not the issue and that the problem lies within the recruitment of β-arrestin or a different process within that pathway.

These investigations have solely focused on two bioluminescence-based assays to study the effects of GPSM3 on cellular signalling. Although my results are internally consistent and clearly point to a reduction in β-arrestin recruitment to G_i-coupled receptors mediated by the ability of GPSM3 to promote Gβγ-related signalling, it would be helpful to extend and further confirm these findings by studying the same phenomenon using alternative methods, as mentioned in the previous section. In addition, the effects of GPSM3 must be studied with other

G_i-coupled GPCRs in different cell types, specifically cell types that naturally contain GPSM3 (THP-1, rat vascular smooth muscle cells) in order to generalize the effects seen.

Results generated in this study solely relied on human cell-based models; however, additional studies would be helpful to further examine the effect of GPSM3 on cellular signalling *in vivo*. There is evidence that $GPSM^{-/-}$ mice exhibit protection against monocyte-driven model of acute inflammatory arthritis. Therefore, it would be interesting to determine whether these differences are a result of $G\alpha_i$-mediated inhibition or promotion of $G\beta\gamma$-mediated signalling. Furthermore, my data suggest that GPSM3 impedes desensitization of G_i-coupled receptors, notably μ-opioid receptor. Activation of this receptor type facilitates analgesia, sedation and euphoria and has been widely targeted by pharmaceutical companies for pain management. Given that GPSM3 reduces β-arrestin recruitment to this receptor, it is possible that GPSM3 could prolong analgesic properties. Thus, it would be valuable to study whether $GPSM3^{-/-}$ mice exhibit lower pain thresholds when compared with wild types controls.

4.5 Conclusions

In conclusion, I have identified that presence of GPSM3 decreases the recruitment of β-arrestin proteins specifically to G_i-coupled receptors. Even though GPSM3 can bind to and inhibit $G\alpha_i$-mediated signalling, it seems that the primary mechanism by which GPSM3 is reducing β-arrestin binding is through a $G\beta\gamma$-dependent pathway. This effect was mimicked by $G\beta_1\gamma_2$ overexpression and rescued using a $G\beta\gamma$-specific inhibitor, GRK2ct/βARKct. In addition, GPSM3 exhibits a net inhibitory effect on endogenous adenylyl cyclase activity in HEK293

cells; however, the mechanism by which this occurs is poorly understood. A better understanding on how GPSM3 alters GPCR signalling is needed to understand its role in pathophysiological signalling and disease states, such as inflammation, rheumatoid arthritis and ankylosing spondylitis, in order to support new therapeutic interventions.

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Appendices

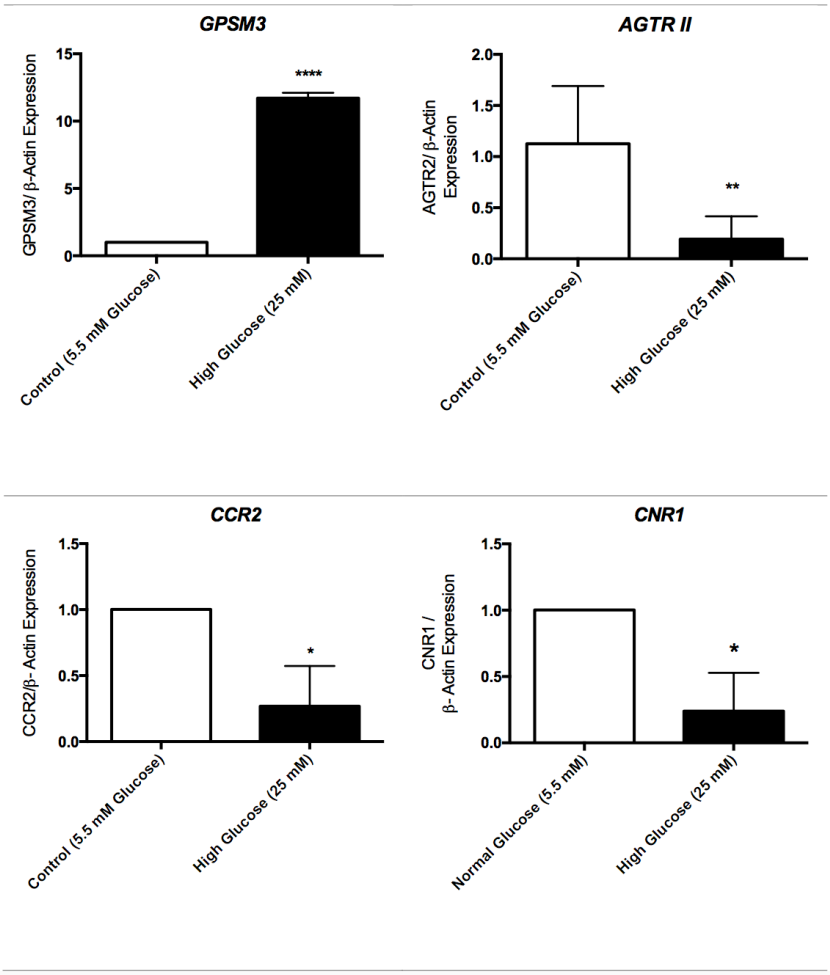

Figure A-1. Expression of *AGTRII, GPSM3, CNR1 and CCR2* mRNA in E11 conditionally immortalized murine podocytes under normal and hyperglycemic conditions. Podocytes were treated with either normal (5.5mM) or high glucose (25mM) levels in medium for 48 hours. RNA was subsequently isolated using Trizol reagent and 1ug of cDNA was plated in triplicate for RT-qPCR analysis using SensiFAST^{TM} SYBR No-ROX Kit. Means between groups were significant *p<0.01, **p < 0.001, ****p<0.0001. T test, n=3

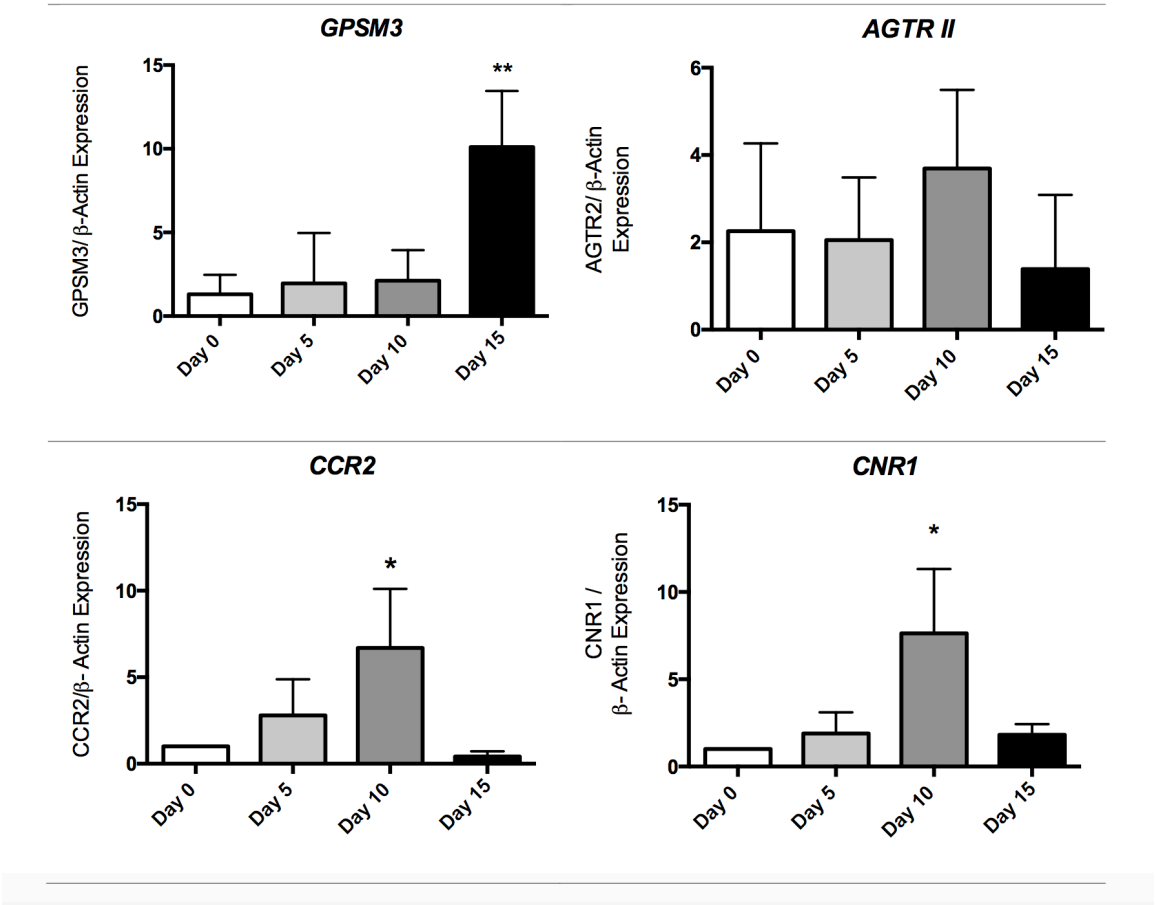

Figure A-2. Expression of *GPSM3, AGTRII, CNR1 and CCR2* mRNA in E11 conditionally immortalized murine podocytes over the course of differentiation. 1ug of cDNA was plated in triplicate for RT-qPCR analysis using SensiFAST™ SYBR No-ROX Kit. Means between groups were significant *$p < 0.05$, **p<0.001 ONE WAY-ANOVA with Bonferroni's test, n=3-6

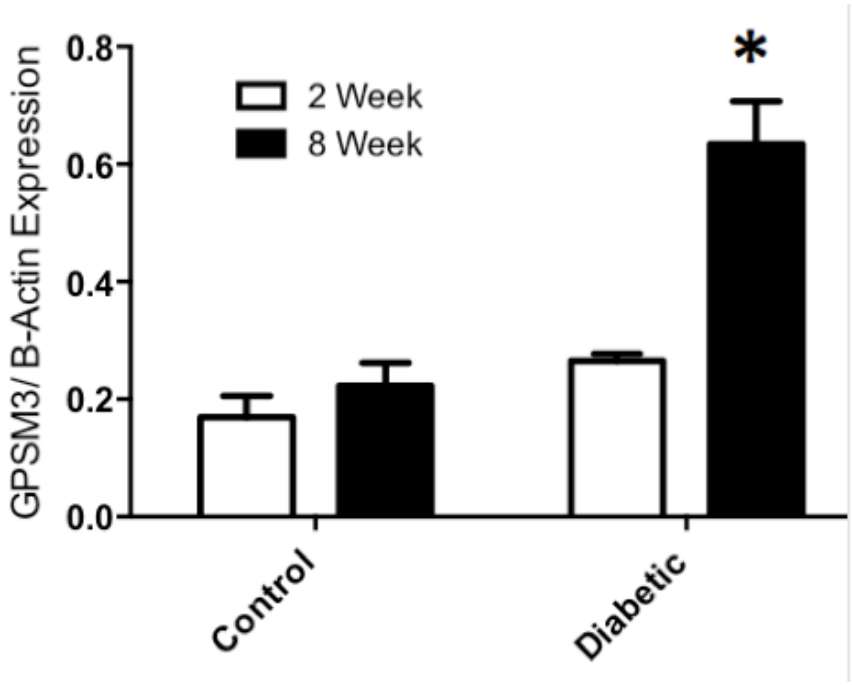

Figure A-3. GPSM3 transcript expression in 2 and 8-week control and diabetic mouse kidney tissues. GPSM3 transcript expression relative to β-Actin in 2 and 8-week control and Streptozotocin-induced diabetic C57BL/6 mice (n=4). 1ug of cDNA was plated in triplicate for qPCR analysis using SensiFAST^{TM} SYBR No-ROX Kit. Means between 2-week control and diabetic mice were not significant. Means between 8-week control and diabetic mice were significant *$p<0.05$, TWO WAY-ANOVA, n=4

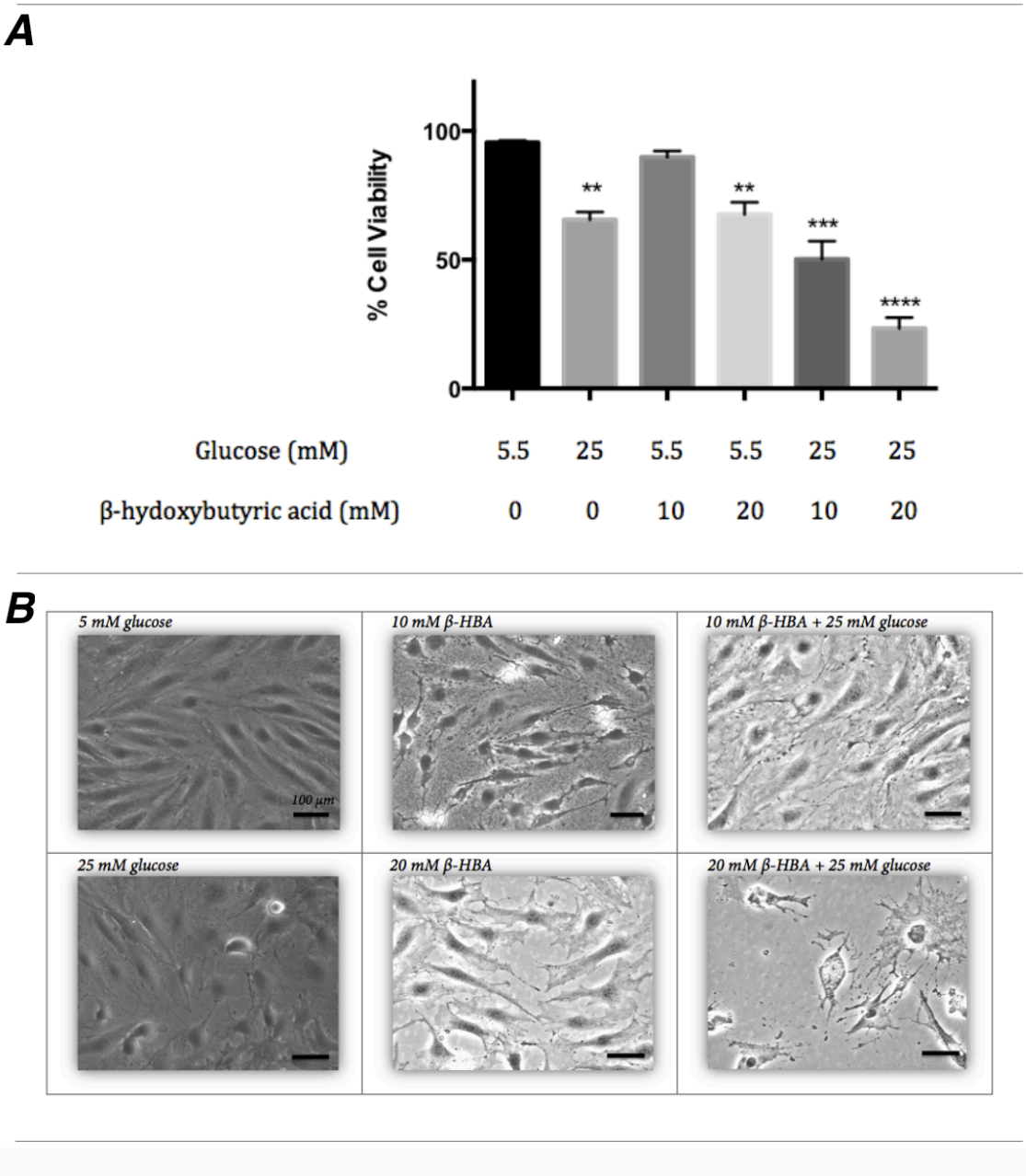

Figure A-4. **Hyperglycemia and β-hydroxybutyric acid reduce cell viability. (A)** Cell viability was determined by the trypan-blue dye exclusion assay after treatment for 24 hours. Data are derived from two independent experiments carried out in duplicate. $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$, ONE-WAY ANOVA with Bonferroni's test, n=2. **(B)** Images are representative of the two independent experiments.

Curriculum Vitae

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Post-secondary Education and Degrees:

The University of Western Ontario
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2011-2016 BMSc.

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Honours and Awards:

Recipient of the William Mahon Award for Best Oral Presentation
Canadian Society of Pharmacology and Therapeutics
May 2018

Oral Presenter
Cell Stress Research Group
Western University
May 2018

CSPT Travel Award
Canadian Society of Pharmacology and Therapeutics
Toronto, Ontario
May 2018

Best Oral Poster Award for Clinical and Basic Pharmacology
Department of Physiology and Pharmacology Research Day
November 2017

CSPT Travel Award
Canadian Society of Pharmacology and Therapeutics
Dalhousie University
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SOGS Teaching Assistant Award Nominee
Western University
2017-2018

Certificate in University Teaching
Western University
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2018

Student Development and Recognition Program Certificate
Lawson Health Research Institute
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Related Work Experience

Graduate Teaching Assistant
Physiology 2130
Western University
2016-2018

Abstract and Poster Judge
Western Student Research Conference
Western University
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Sports Representative
Physiology and Pharmacology Graduate Student Council
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Graduate Teaching Assistant Departmental Steward
PSAC Local 610
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Schulich Committee Representative
Schulich Graduate Student Council
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Seminar Speaker
Talks on Fridays
Lawson Health Research Institute
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March 2018

Pharmacology Representative
Inspiring Young Women in STEM Conference
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Conferences Attended:

2016 – Great Lakes GPCR Retreat (Chicago, IL, USA)
2016 – Department of Physiology and Pharmacology Research Day (London, ON)
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2017 – Department of Medicine Research Day (London, ON)
2017 – Canadian Pharmacology and Therapeutics Conference (Halifax, NS)
2017 – Great Lakes GPCR Retreat (Ottawa, ON)
2017 – Department of Physiology and Pharmacology Research Day (London, ON)
2018 – Women in STEM Conference (London, ON)
2018 – London Health Research Day (London, ON)
2018 – Department of Medicine Research Day (London, ON)
2018 – Canadian Pharmacology and Therapeutics Conference (Toronto, ON)

Publications:

Surmanski AA. Understanding therapies selected for managing pain associated with spinal cord injuries. *UWOMJ* 87(1): 25-27

Masciantonio MG and **Surmanski AA**. Medical smartphone applications: A new and innovative way to manage and treat health conditions from the palm of your hand. *UWOMJ* 82(2): 51-53