

Differential Glycosylation of the Inwardly Rectifying Potassium Channel Kir7.1
by G protein-coupled Receptors

By

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Dedicated to my son,
Theodore Jared Levi Carrington

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CHAPTER I

PROTEIN-PROTEIN INTERACTIONS OF INWARD RECTIFYING POTASSIUM CHANNELS

Introduction

Inward rectifying potassium (Kir) channels are one of the largest groups of ion channel families found in mammalian cells. This group of membrane proteins plays various roles in physiology from potassium channel transport in the kidney to controlling excitability within neurons. So essential is the function of these membrane proteins, that mutations within the family are linked to various human diseases (Abraham, Jahangir et al. 1999, Hejtmancik, Jiao et al. 2008). Few drugs directly target these Kir, however these channels often act in concert with other proteins in the cell such as G-protein coupled receptors (GPCRs). These interacting proteins can modulate channel function to play various roles in physiology, with some being indeed druggable. Here we explore these ion channels and protein-protein interactions that regulate their function in physiology.

Molecular structure and function of potassium inward rectifiers

Kirs are a subgroup of potassium channels comprised of two transmembrane domains (Fig 1.1A). These transmembrane domains are linked by an extracellular region which form the pore domain, with both the amino and carboxyl terminal of the channel intracellularly located. The Kir family is composed of 16 channels from three different subgroups; potassium transporters (Kir1.x, Kir7.1, Kir4.x, Kir5.1), classical Kir channels (Kir2.x), G-protein gated K⁺ channels (GIRK, Kir3.x) and ATP-sensitive K⁺ channels (Kir6.x) (Hibino, Inanobe et al. 2010) (Fig 1.1B).

Individual Kir subunits assemble as a tetramer to form a functional ion channel (Fig 1.1A). Channels typically form homo-tetramers, except Kir5.1 which assembles with Kir4.1 in order to form a functional channel (Konstas, Korbmayer et al. 2003, Hibino, Fujita et al. 2004). Kir2.x exist as homo-tetramers but can also form hetero-tetramers with other family subunits (Gernot, Peter et al. 2002, Lior, Lisa et al. 2011).

Pore domain and selectivity filter

The pore domain of the channel, referred to as the H5 region, is comprised of the ion selectivity filter with the sequence T-X-G-Y-G, which is shared with other potassium selective ion channels (Doyle, Cabral et al. 1998, Shealy, Murphy et al. 2003). In addition to the selectivity filter, Kir channels also share an arginine residue nearby G-Y-G-X-**R**-X, the absence of which leads to smaller currents as seen in Kir7.1, that has a methionine present in its pore (G-Y-G-X-M-X) (Doring, Derst et al. 1998). Replacement of methionine 125 in Kir7.1 with arginine allows larger currents from these channels. This pore domain is also known to be susceptible to blockade by intracellular cations such as Mg²⁺ and polyamines (Kurata, Cheng et al. 2011).

Post-translational modifications

Inward rectifiers possess classical sites for post translation modification such as phosphorylation and glycosylation. Protein kinase A (PKA) and protein kinase C (PKC) can modulate the activity of these channels by phosphorylation of serine or tyrosine residues located in the C-terminal tail. Phosphorylation can have various effects on channel function. For example, phosphorylation of Kir6.2 by PKA enhances channel activity (Béguin, Nagashima et al. 1999).

Additionally, phosphorylation can also affect protein trafficking; for example, Kir1.1 requires PKC phosphorylation for surface expression (Lin, Sterling et al. 2002).

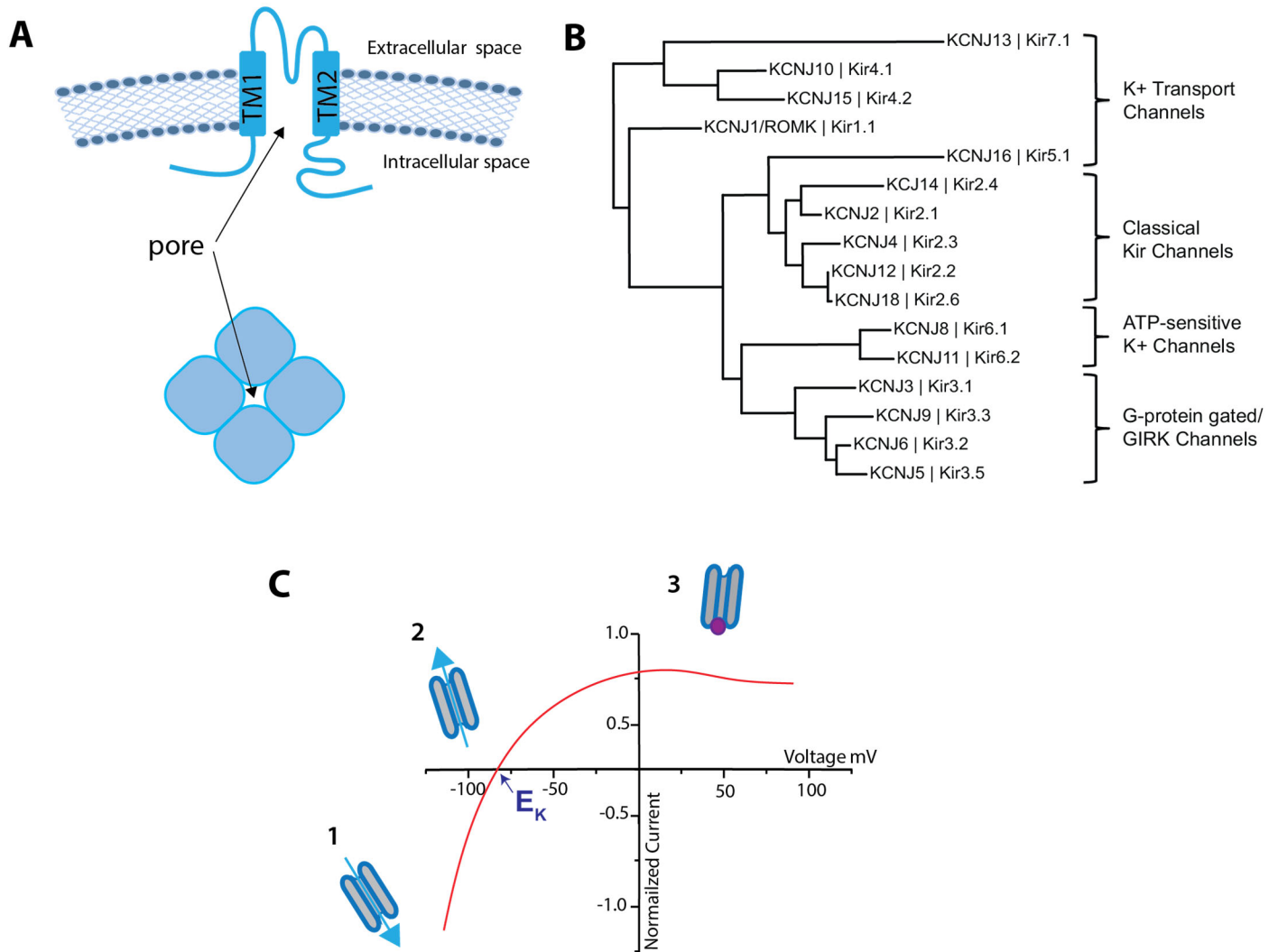


Figure 1.1 Structure and function of inwardly rectifying potassium channels

A. Schematic of an individual Kir channel subunit with two transmembrane domains TM1 and TM2. Channel subunits assemble as functional tetramers forming a single pore in the center. **B.** The Kir family is composed of 16 different subtypes classified into four groups. The phylogenetic tree was generated using online phylogeny software (Dereeper, Guignon et al. 2008). **C.** Representative current-voltage (I/V) curve of a Kir channel. At negative potentials potassium flows into the cell generating an inward current (1). When the Nernst (E_K) potential for potassium is reached, Kirs allow the outflow of potassium ions as an outward current (2). As the membrane becomes more positive to E_K the pore becomes blocked by cations or polyamines in a process known as rectification.

Kir channels can also undergo glycosylation which plays a role in the function of some channels. Kir channels exhibit N-linked glycosylation, with no reports of O-linked glycosylation in literature. Kir1.1 and Kir7.1 require glycosylation for effective function, as reduced open channel probabilities are observed in glycosylation resistant mutants (Schwalbe, Wang et al. 1995, Carrington, Hernandez et al. 2018). While other channels are observed to be glycosylated such as Kir4.1, roles for glycosylation remain unclear in these channels. Putative glycosylation sites are located in extracellular loops of the channel which may suggest a functional consequence on channel activity.

Binding domains

Inward rectifiers can be regulated by the binding of lipids or other proteins directly to their subunits. Kir channels have a phosphatidylinositol 4,5-bisphosphate (PIP₂) binding site which is essential for channel gating (Stansfeld, Hopkinson et al. 2009, Hansen, Tao et al. 2011). This site is located between the transmembrane domain and the interface of the C-terminal domain (Hansen, Tao et al. 2011). The binding of PIP₂ aids channel gating, and its depletion leads to “channel rundown” with a decline in activity. This activity can be rescued if PIP₂ is replenished within the cell.

These channels also have binding sites for adenosine triphosphate (ATP), with K_{ATP} channels widely shown to be most sensitive (Craig, Ashcroft et al. 2008). K_{ATP} channels are comprised of Kir6.x and the unrelated sulfonylurea receptor subunits (SUR) (Martin, Yoshioka et al. 2017). These complexes are inhibited by ATP binding to cytoplasmic regions within Kir6.x (Antcliff, Haider et al. 2005, Craig, Ashcroft et al. 2008), with ATP binding to SUR as well. The protein-protein interaction of SUR1 and Kir6.x is discussed in more detail later in this chapter.

Channel function

Kir channels selectively carry potassium ions across the membrane of the cell with changes in membrane potential. These channels respond to changes in voltage across the cell, but they lack the positively charged S4 transmembrane voltage sensing domain found in classical voltage gated ion channels. The movement of ions into the cell is called inward current, and out of the cell as outward current (Fig 1.1C). These channels have larger inward currents due to their rectification properties. Simply, at membrane potentials negative to the equilibrium potential of potassium (Nernst potential or E_K), Kir channels allow the flow of potassium ions into the cell (Fig 1.1C-1). As the membrane potential becomes more positive to E_K , potassium ions flow out of the channel generating outward currents (Fig 1.1C-2). These outward currents are limited as intracellular cations (Mg^{2+}) and polyamines block the outward flow of potassium as the membrane potential becomes more positive (Fig 1.1C-3). The blockade of additional outward currents at positive potentials is termed “inward rectification” (Lu 2004).

Regulation of inward rectifiers by protein-protein interactions

Kir channels can be regulated by direct protein-protein interactions which control pore function. Current known interactors are various receptor subunits, G-proteins and anchoring/scaffolding proteins such as PSD95 (Fig 1.2). These proteins will be explored in more detail in this section.

Receptor subunits

The sulphonylurea receptor (SUR) is an ATP-binding cassette (ABC) transporter that is part of a large group of membrane proteins. These proteins use ATP hydrolysis to generate energy

to transport substances across the cell membrane (Aittoniemi, Fotinou et al. 2009). Whereas ABC transporters function as independent units to transport molecules across the membrane, the sulphonyl urea class of receptors are coupled to the Kir6.x ion channel to form the K_{ATP} channel. SURs assemble 1:1 with Kir6.x subunits to form an octameric complex. While there are two types of SUR- SUR1 and SUR2, SUR1 assembles with Kir6.2 to form the K_{ATP} channel in beta cells of the pancreas (Li, Wu et al. 2017). SUR1 expression is also required for Kir6.2 surface expression, due to an endoplasmic reticulum (ER) retention signal on the channel which requires SUR for release and transport to the membrane (Zerangue, Schwappach et al. 1999). SURs are composed of 3 transmembrane domains (TMD0-TMD2) which have 4-6 transmembrane helices. The intracellular loop of the receptor between TMD1 and TMD2 and the C terminal tail form two nucleotide binding domains (NBDs) that sense changes in intracellular nucleotides. SUR is directly coupled to Kir6.2 by the intercellular loop between TMD0 and TMD1 interacting with 7 residues on the N terminal of Kir6.2 (Li, Wu et al. 2017). Interestingly, it was recently shown that the binding of PIP2 to Kir6.2 can uncouple the interaction of SUR and the channel (Li, Wu et al. 2017). The PIP2 binding site partially overlaps with the SUR1/Kir6.2 interacting region, which was revealed by Cryo-EM.

Within beta cells when glucose levels are low, MgADP binds to the NBD of SUR causing Kir6.2 to open which hyperpolarizes the cell and reduces insulin release (Aittoniemi, Fotinou et al. 2009). When glucose levels are high causing increased ATP in the cell, ATP binds directly to Kir6.2 leading to channel closure, membrane depolarization and insulin release. The sensitivity of SUR1 to glucose levels has allowed this system to be targeted in the treatment of diabetes. Glibenclamide is a widely used diabetes drug that binds to and inhibits SUR1 by inducing an inactive conformation (Proks, Reimann et al. 2002). SUR is thereby unable to respond to changes

in MgADP leaving Kir6.2 in the closed state. This allows the cell to depolarize promoting insulin release. K_{ATP} channels also exist in muscles and cardiac cells (Gribble, Tucker et al. 1998). Thus, Kir6.2 channels can be regulated directly by a receptor to play a key role in cellular function.

The cystic fibrosis transmembrane conductance regulator (CFTR) is another ABC transporter shown to form weak K_{ATP} channels with a splice variant of Kir1.1 in the Kidney (Ruknudin, Schulze et al. 1998). Kir1.1 channels are involved in potassium transport and different isoforms are expressed along the nephron (Kir1.1a-c)(Welling and Ho 2009). Each isoform has a different physiological function. CFTR assembles with Kir1.1b and bestows weak sulphonyl urea and ATP sensitivity to the channel. Unlike the SUR/Kir6.2 K_{ATP} channel, the interaction of CFTR and Kir1.1 appears to be dependent on Na/H exchange regulatory factors (NHERF1/2) which serve as a scaffolds to support CFTR/Kir1.1 coupling (Yoo, Flagg et al. 2004). The mechanism of CFTR inhibition of Kir1.1 is thought to be indirect and not clearly understood, but PKA mediated phosphorylation of the channel uncouples the interaction and relieves ATP-dependent inhibition. Unlike Sur1 and Kir6.2, Kir1.1 does not require CFTR for surface expression.

GPCRs

G-protein coupled receptors (GPCRs) are one of the largest druggable classes of proteins in therapeutics. These seven transmembrane domain receptors regulate a wide range of functions in all tissues. GPCRs transduce signals within a cell via effectors called G-proteins which activate various signaling pathways. In a broad sense, GPCRs can activate Galpha ($G\alpha$) subunits which can be stimulatory (G_s) proteins or inhibitory (G_i) proteins and other classes such as G_q . $G\alpha$ proteins exist in a trimeric complex with Gbeta ($G\beta$) and Ggamma ($G\gamma$) proteins. Activation of the receptor leads to activation of the $G\alpha$ subunit causing the release of the $G\beta\gamma$ complex to mediate

their effects (Smrcka 2008). Kir channels are typically regulated by GPCRs via G $\beta\gamma$ complexes (Hommers, Lohse et al. 2003). As such, G-protein inwardly rectifying potassium channels (GIRKs) are activated by G proteins after receptor activation. GIRK channels are coupled through Gi linked GPCRs such as M2 muscarinic receptors in the heart (Wang, Liang et al. 2013). These GPCRs can directly associate in complex with Kir channels even though they couple indirectly (Lavigne, Ethier et al. 2002). To date only the melanocortin-4 receptor (MC4R) has been reported to signal through Kir7.1 channel independently of G proteins (Ghamari-Langroudi, Digby et al. 2015, Anderson, Çakir et al. 2016) (see appendix). The exact mechanism of interaction remains unknown. Outside of this, studies have been published with engineered GPCR-Kir subunits to create ligand-gated Kir channels (Moreau, Revilloud et al. 2017). In these proteins, the GPCRs mechanically transduce their signal through the N-terminal of the Kir channel to cause channel opening or closing. The length of the N terminal region of the channel and the GPCR may be altered to activate or inhibit the system. This allows channel activity to be used as an artificial read out of GPCR activity and may suggest a possible mechanism for the MC4R-Kir7.1 interaction.

GPCRs can also regulate the trafficking of Kir channels to control function. The calcium sensing receptor (CaR) is a GPCR expressed in the kidney that regulates salt and water transport. CaR was found to interact with Kir4.1 and Kir4.2 and regulate channel activity by reducing its surface expression (Cha, Huang et al. 2011). The reduction in Kir4.1 surface expression was proportional to the activity of the GPCR, in that non-functional GPCRs failed to have an effect on the channel. This effect was mediated by G protein G α_q and caveolin-1 dependent internalization pathways. The mechanism by which G α_q leads to caveolin-1 mediated internalization, however, remains unclear. GIRK channel surface expression can also be regulated by the GPCRs to which they couple and form complexes. GPCR signaling is generally switched off by receptor

phosphorylation and the recruitment of beta arrestin proteins. Beta-arrestins lead to the recruitment of clathrin, a vesicle membrane coat protein that mediates endocytosis. GIRK channels have been observed to co-internalize with the delta opioid GPCR following receptor desensitization (Nagi, Charfi et al. 2015). GPCRs can thus regulate GIRK channels through G protein mediated activation, and removal of channels from the membrane.

G-proteins

GIRK channels are the Kir3.x subfamily whose activity is coupled directly to G-protein signaling. D₂ and M₂ GPCRs are known to couple to GIRK channels in the brain and in the case of M₂, in other tissues like the heart (Harvey and Belevych 2003, Huang, Griffin et al. 2013). GPCRs that couple to GIRKs are predominantly G_i coupled receptors. As described previously, GIRK channels are activated by Gβγ proteins which are released from the complex after GPCR activation. The exact mechanism is unknown, but it is thought that residues in the N and C terminal domains of the channel interact with the Gβγ complex to mediate channel activation (He, Yan et al. 2002).

Interestingly, while not a classical GIRK channel, the Kir2.3 channel has been reported to be inhibited by Gβγ (Cohen, Sha et al. 1996). This is divergent from GIRK channels which are activated by the same G protein complex. The only known mechanism by which G-proteins inhibit Kir channels, is by GPCR activation of G_q which leads to depletion of the essential lipid PIP₂ from the membrane. G_q activates phospholipase C, which converts PIP₂ to diacylglycerol (DAG), and inositol triphosphate (IP₃) (Kobrinisky, Mirshahi et al. 2000). As stated earlier, depletion of PIP₂ leads to channel rundown and a reduction in channel activity. Thus, the mechanism of Gβγ

inhibition of Kir2.3 needs to be further explored as it was studied by expressing both proteins in *Xenopus* oocytes.

An additional mechanism of Gq mediated inhibition of Kir2.1-2.3 channels was reported *in vitro*. Activation of the M1 receptor was found to reversibly inhibit all Kir2 subtypes. When cells were loaded with guanosine 5'-O-(2-thiodiphosphate) GDP β S which blocks – GTPase hydrolysis, the effects of M1 receptor activation was attenuated. At this point classical knowledge would suggest G β γ signaling as shown in Fig 1.2, however the study found that the small GTPase Rho mediated the inhibition of the Kir2.x channel (Jones 2003, Rossignol and Jones 2006). Transfection of an activated Rho GTPase reduced current density, which could be inhibited by a selective inhibitor of Rho, exoenzyme C3 transferase from *Clostridium botulinum*. This mechanism was found to be calcium dependent, and it is thought that M1 mediated calcium release leads to Rho GTPase activation and thus inhibition of Kir2.x channels. The study however did not carefully link calcium release to Rho activation, and only chelators of calcium were used to show diminished channel activity. The M1 Gq mediated release of calcium occurs through PLC generation of IP3 which activates calcium channels on the endoplasmic reticulum. Inhibitors of PLC would contribute to further establishing a link between M1 and Kir2.x channels. Additionally, Rho GTPases are a family of small guanine nucleotide binding proteins of which Ras, Cdc42/Rac and RhoA are extensively studied. The inhibitor of Rho, exoenzyme C3 transferase, used in the study blocked all Rho GTPases. Cdc42/Rac is reportedly activated by elevation of intracellular calcium within HEK293T cells (Jin, Guan et al. 2005). It is therefore likely that Kir2.x channels are inhibited specifically by Cdc/Rac which has not been directly tested. Rho GTPases also do not require receptor activation for function, so other activities in the cell which upregulates Rho

GTPase signaling can also affect Kir2.x channel activity. Whether Rho GTPases have an effect on other Kir family channels is unknown.

Other proteins have also been reported to interact with GIRK channels when found in complex with GPCRs. The GABA_BR GPCR signals to GIRK channels within neurons of the hippocampus. GABA_BR-GIRK signaling can be negatively modulated by regulators of G protein signaling (RGS) proteins (Olga, Keqiang et al. 2014). These proteins have been shown to increase the rate of G protein inactivation. Specifically, loss of RGS7 was found to result in a slower rate of deactivation of GIRK channels. Additionally, loss of R7BP, a membrane targeting RGS subunit caused additional reductions in channel deactivation. RGS7 has also been shown in vitro to exist in complex with GIRK channels (Zhou, Chisari et al. 2012). The RGS7 and R7BP proteins require the atypical G-protein G β 5 for function. Loss of G β 5 also leads to loss of RGS7 expression in mice (Xie, Allen et al. 2010). G β 5 has been shown to form complexes with RGS7, and GIRK1. G β 5 is able to bind to all GIRKs except GIRK4 which is typically found in the heart. Specifically, while RGS7/ G β 5 forms complex with GIRK channels, the presence of R7BP enhances formation of the complex (Zhou, Chisari et al. 2012). R7BP is thought to be a scaffold by some, where the palmitoylated protein is anchored to the membrane supporting the RGS7/G β 5 interaction with the channel (Zhou, Chisari et al. 2012). The binding site of G β 5 on GIRKs is similar to that of G β γ , but both the RGS7/G β 5 complex and G β γ can both bind non-competitively to the channel (Zhou, Chisari et al. 2012). Thus, control of GIRK channel activation kinetics is regulated by the RGS proteins and G β γ , which both interact directly with the channel.

Scaffolding proteins and other interactions

In neurons, many ion channels are located at synaptic densities. The trafficking of proteins to these regions is supported by anchoring and scaffolding proteins. These anchoring proteins interact with the cellular cytoskeleton to target membrane proteins to key areas of the cell. Many of these anchoring proteins typically contain PDZ domains. PDZ domains are small regions consisting of beta-stranded and 2 or 3 alpha helical protein structures (Lee and Zheng 2010). These regions serve as binding sites for target proteins which are being “anchored” to a discrete region of the cell. One such anchoring protein is postsynaptic density protein 95 (PSD-95). PSD-95 is found in the post synaptic densities of neurons as the name suggest and is crucial to the clustering of channels and receptors at the post synaptic membrane. Loss of PSD-95 leads to a breakdown of the post synaptic density (Chen, Nelson et al. 2011). Kir2.3 ion channels have been observed by electron microscopy in the post-synaptic density of dendritic spines (Inanobe, Fujita et al. 2002). It was later found that Kir2.1 and Kir2.3 co-localize with PSD-95 in hippocampal neurons (Cohen, Sha et al. 1996). The C -terminal tail of the ion channel was found to be critical for this interaction. In the presence of PSD-95 these ion channels were observed to form clusters within the cell. Phosphorylation of Kir2.3 by PKA at a C terminal serine dissociates the interaction with PSD-95 which suggest regulation of the interaction (Cohen, Sha et al. 1996). Kir5.1, which typically requires co-assembly with Kir4.1 to form functional channels, was also observed to colocalize with PSD-95 at the postsynaptic density in neurons (Tanemoto, Fujita et al. 2002). In cultured cells, when Kir5.1 was co-expressed with PSD-95, functional homomeric channels were formed which clustered at the membrane. In the absence of PSD-95 channels were mostly distributed intracellularly. It was further shown that PSD-95 facilitates surface expression of Kir5.1. Indeed, as Kir5.1 channels are typically found as hetero-tetramers with Kir4.1, Kir4.1 expression has also

been shown to induce Kir5.1 localization at the membrane (Tanemoto, Abe et al. 2014). Thus, Kir5.1 is able to form functional channels in the absence of Kir4.1 when PSD-95 is expressed.

Anchoring proteins also function in other cells besides neurons to target Kir channels. The Channel-interacting PDZ domain protein (CIPP) expressed in the brain and kidney was found to interact with Kir4.1 channels and to promote clustering at the membrane (Kurschner, Mermelstein et al. 1998). Kir4.1 channel activity doubled when CIPP was co-expressed in cultured cells. Membrane associated guanylate kinase with inverted domain structure-1 (MAGI-1) is another anchor protein essential to basolateral membrane expression of Kir4.1/5.1 channels in tubular cells of the kidney (Tanemoto, Abe et al. 2014). Mutations in Kir4.1 that lead to renal salt wasting dissociate MAGI-1 from Kir4.1/5.1 channels reducing basolateral membrane expression.

Kir 2.3 interacts with tax-interacting protein-1 (TIP-1) in concert with Lin-7. These two PDZ domain anchoring proteins regulate trafficking of the channel from the membrane to the endocytosis pathway (Yan, Zhou et al. 2009). Likewise, Kir1.1 was found to interact with PDZ domain containing Na/H exchange regulatory factors (NHERF1/2) in the kidney (Yoo, Flagg et al. 2004). NHERF increases the surface expression of Kir1.1 and facilitates coupling with CFTR in formation of CFTR/Kir1.1 K_{ATP} channels.

As mentioned previously, Kir phosphorylation occurs via PKA or PKC. The targeting of PKA to subcellular locations occurs by interaction with A Kinase-anchoring proteins (AKAPs). Kir2.1 channels have been shown to associate with AKAP79/5 at the membrane (Dart and Leyland 2001). AKAPs thereby maintain kinase substrates in close proximity which allows efficient tuning of ion channel activity.

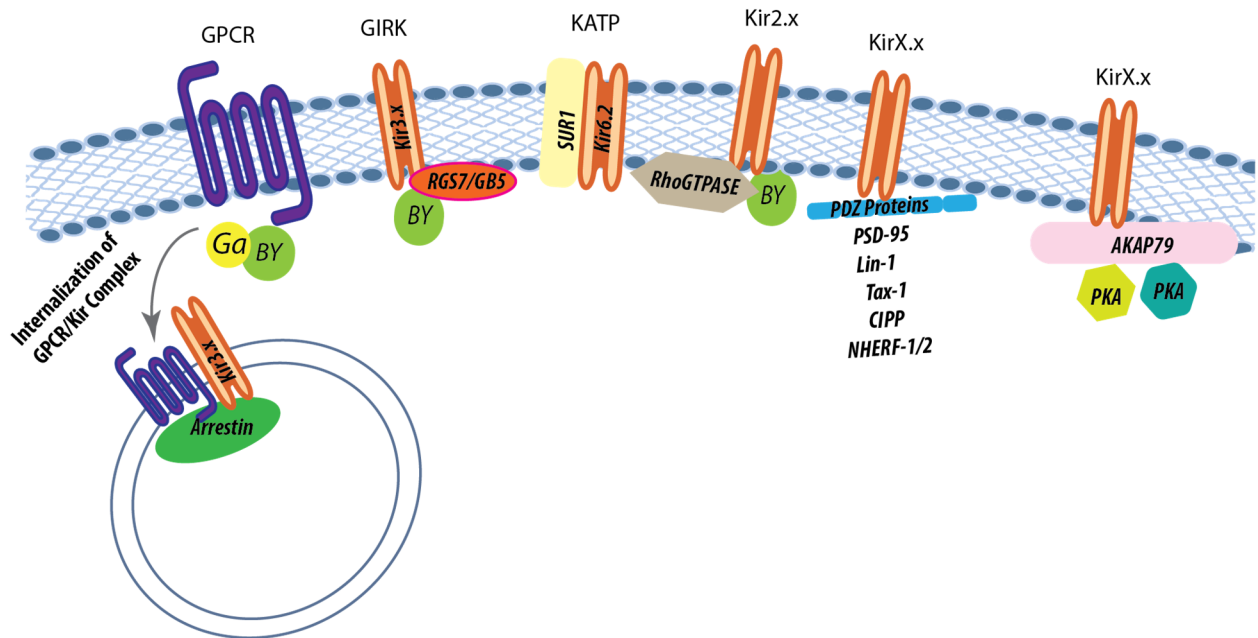


Figure 1.2 Known protein-protein interactions of inward rectifying potassium channels.

Diagram depicts various proteins known to interact with different Kir channels and their relative cellular locations. GPCRs typically couple to GIRK channels which are activated by $G\beta\gamma$ and tuned by the RGS7/ $G\beta 5$ complex. When GPCRs are switched off, GIRK channels can also be internalized with the receptor by arrestin binding and recruitment of clathrin (not shown). KATP channels are composed of Kir6.2 and the SUR1 receptor. Kir2.x can also be regulated $G\beta\gamma$ to cause channel closure, and also by RhoGTPase. Kir channels also interact with scaffolding proteins through interaction with PDZ domains present on many of these proteins (listed). These proteins support trafficking of different channels through the cell (see section “scaffolding proteins and other interactions”). AKAPs also associate with Kirs to maintain PKA and PKC in close proximity. These phosphorylate the channel to regulate activity.

MC4R, the first G protein-coupled receptor reported to functionally interact with Kir7.1

Energy homeostasis is an essential physiological function that is tightly regulated. It controls the drive for food intake, and also promotes energy expenditure. Dysregulation in this system can lead to obesity or symptoms of anorexia. Central to the control of energy balance are the melanocortin peptides which act on MC4 receptors throughout the CNS (Poggioli, Vergoni et al. 1986). Specifically, these peptides cleaved from the precursor protein proopiomelanocortin (POMC), are endogenous agonist for the G protein-coupled melanocortin receptors. Within the family of five melanocortin receptors, the MC4R plays key roles in energy homeostasis (Cone 1999). MC4R Knockout mice were found to display early onset obesity with increased linear growth, hyperphagia and decreased energy expenditure (Huszar, Lynch et al. 1997). It was also shown that MC4R displays gene dosage effects, wherein mice with one copy of the gene display an intermediate phenotype. MC4R's role in energy homeostasis is also highly conserved across multiple species (Logan, Bryson-Richardson et al. 2003, Aspiras, Rohner et al. 2015). Ultimately, MC4R was found to regulate feeding, where activation of the receptor by its endogenous agonist α -MSH reduced the drive for food intake, and increased energy expenditure. The molecular mechanisms governing the signaling of MC4R is of keen interest, providing a possible avenue for obesity and cachexia treatments.

MC4R was first cloned in 1993, based on homology to previously discovered family receptors MC1R and MC2R (Mountjoy, Robbins et al. 1992, Gantz, Konda et al. 1993). The receptor was profiled and found to couple to the G stimulatory protein ($G_s\alpha$), which leads to the downstream upregulation of cAMP (Fig 1.3). An endogenous inverse agonist agouti-related peptide (AgRP) acts to inhibit the receptor and reduce cAMP generation. GPCRs have also been shown to signal to different G proteins and can thus activate different signaling pathways. As

MC4R activation leads to multiple physiological effects, whether Gs α mediates all these responses has been the goal of many studies. Deletion of Gs α within MC4R neurons mimicked the MC4R knockout obesity phenotype with hyperphagia and increased food intake (Podyma, Sun et al. 2018). Gq α and G11 α are two other heterotrimeric G proteins which activate phospholipase C and increase intracellular calcium levels (Fig 1.3). Calcium can act as a second messenger within neurons with multiple downstream effects. Deletion of Gq α and G11 α from the paraventricular nucleus of the hypothalamus (PVN) where MC4R controls food intake, also led to obesity, hyperphagia and increased linear growth (Li, Shrestha et al. 2016). Interestingly, an MC4R agonist Melanotan-II (MTII) was unable to reduce food intake in PVN Gq/11 KO mice, but did reduce food intake in PVN GsKO mice. Additionally, MC4R affects sympathetic nervous system output, with agonist activation increasing blood pressure. In PVN Gq/11 KO mice, MTII increased systolic blood pressure, whereas it failed to have a response in PVN Gs KO mice (Li, Shrestha et al. 2016). These data indeed suggest that multiple signaling pathways are activated after MC4R stimulation by α -MSH, which then mediate different physiological effects.

GPCRs like MC4R also lead to arrestin recruitment after receptor activation and G-protein signaling. The binding of arrestins to the phosphorylated C terminal region of the receptor switches off further G-protein signaling. Arrestins not only target the GPCR for internalization, but also facilitate G-protein independent receptor signaling (Luttrell, Gesty-Palmer et al. 2010). Arrestins can couple to the mitogen activated kinase pathway (MAPK), that signals downstream to effects like transcriptional regulation. Biased ligands are actively being developed that can stimulate MC4R to signal via the G protein only or arrestin only pathways. Given the various downstream effects of the receptor, these tools can be useful to differentiate the physiology associated with a particular signaling pathway.

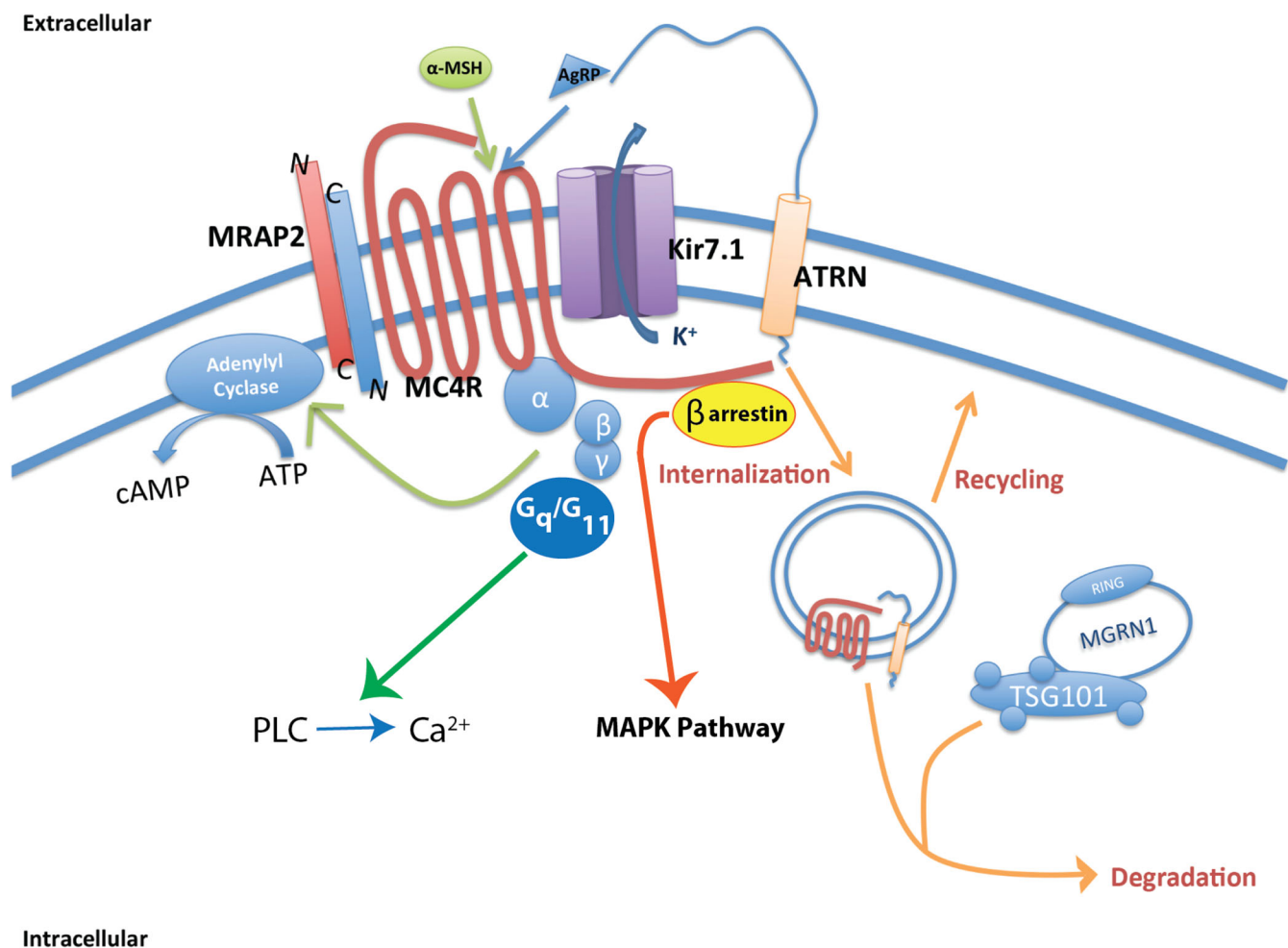


Figure 1.3 Signaling pathways and interacting proteins of MC4R

α -MSH and AgRP binding to MC4R lead to the activation of different signaling pathways. The G protein and arrestin signaling pathways of the receptor are shown. Additionally, MC4R is known to interact with the melanocortin 2 receptor associated protein 2 (MRAP2). Attractin, mahogunin (MGRN1) and tumor suppressor gene 101 (Tsg101) involved in receptor internalization are also shown. PLC- phospholipase C. Figure adapted and modified from Anderson et al 2016, Journal of Molecular Endocrinology. Original figure created by Taneisha Gillyard. Reproduced with permission. See appendix page 89.

MC4R neuronal circuitry has been mapped within the hypothalamus and other regions of the brain. MC4R expression in the central nervous system (CNS) extends to both the sympathetic and parasympathetic systems (Sohn, Harris et al. 2013). In the brainstem, MC4R activity was linked to obesity related hypertension and increased sympathetic activity (Hill and Faulkner 2017). Indeed, loss of MC4R in mice was also recently shown to be associated with cardiomyopathy (Litt, Okoye et al. 2017). Many small molecules targeting MC4R to treat obesity have shown promise in mouse studies, but failed in the clinic due to a pressor side effect (Greenfield, Miller et al. 2009). However, setmelanotide (RM-493) a cyclic peptide has been identified in clinical studies to avoid the pressor side effect (Chen, Muniyappa et al. 2015). This compound maintains MC4R stimulatory activity and mediates modest weight loss (Collet, Dubern et al. 2017). At the molecular level, the mechanism by which setmelanotide can mediate some effects of MC4R but not others is yet to be determined. If individual pharmacological signaling pathways of the receptor can be mapped to specific physiological responses, the case for biased ligands becomes increasingly clear to treat specific disruptions in energy balance.

To study the role of MC4R within the PVN, Ghamari-Langroudi et al recorded from MC4R expressing neurons. They noticed that bath application of α -MSH led to robust neuronal depolarization, with the endogenous inverse agonist AgRP hyperpolarizing the neuron. GPCRs are known to mediate neuronal signaling by coupling to ion channels on the cell membrane. Particularly, inwardly rectifying potassium channels like Kir3.x channels can be activated by GPCRs via G proteins to control neuronal activity. However, using a panel of inhibitors against Kir channels, Kir7.1 was identified as the inward rectifier mediating α -MSH induced neuronal depolarization. Specifically, MC4R activation leads to Kir7.1 closure causing neuronal depolarization, whereas AgRP promotes channel opening leading to membrane hyperpolarization.

MC4R mediated closure of Kir7.1 was also shown to occur independently of G proteins (Fig 1.4A). This finding was indeed surprising, and it identified a potential new mechanism of GPCR signaling to ion channels. Kir7.1 does not fall under the category of G protein inwardly rectifying potassium channels (GIRK, Kir3.x) which typically couple to GPCRs. Thus, the exact mechanism of interaction is unknown.

With Gs signaling shown in KO studies to be essential to MC4R's role as an energy rheostat, the physiological role of MC4R-Kir7.1 signaling was yet to be determined. Anderson et al created mice with MC4R specific deletion of Kir7.1 within hypothalamic neurons. This method was needed as global Kir7.1 knockout is embryonically lethal (Zhong, Chen et al. 2015). In PVN slice preparations, α -MSH failed to induce neuronal depolarization in mice lacking Kir7.1 in MC4R expressing cells (Fig 1.4B). This experiment confirmed the Kir7.1 dependent signaling pathway of MC4R. Phenotypic profiling also showed that these mice had late onset weight gain compared to WT controls, though not as significant as MC4R KO mice (Fig 1.5). Additionally, these mice also had an attenuated response duration to an MC4R peptide agonist LY2112688 to reduce fast induced refeeding compared to controls. These results support a role for the Kir7.1-MC4R pathway in energy homeostasis. With this new data, ultimately three pathways of MC4R have been physiologically profiled (MC4R-Gs, MC4R-Gq/G₁₁, MC4R-Kir7.1).

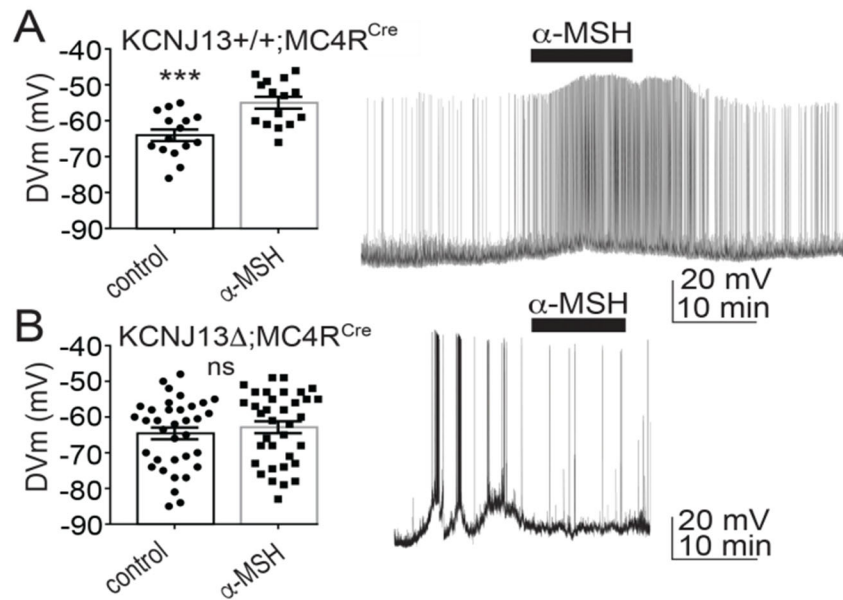


Figure 1.4. MC4R specific deletion of Kir7.1 in PVN neurons leads to defective α -MSH induced depolarization.

Electrophysiology recordings from slices of PVN MC4R-GFP positive neurons. **A.** Recordings from control mice expressing Kir7.1 (KCNJ13), with bath application of α -MSH inducing action potentials. **B.** Slices from $KCNJ13^{\Delta}MC4R^{Cre}$ mice display significantly diminished action potentials when α -MSH is applied. Bar graph represents mean of 15-35 cells ($P < 0.0001$). Data adapted from Anderson et al 2018, Journal of Neuroendocrinology. Reproduced with permission.

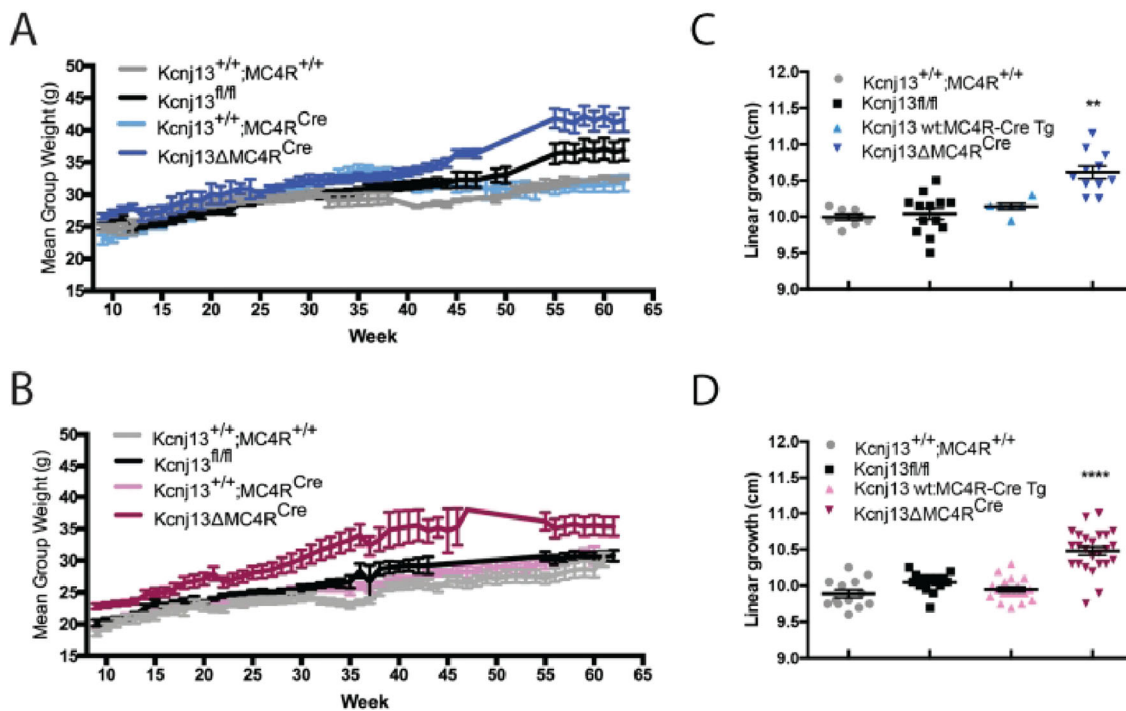


Figure 1.5. Mice lacking Kir7.1 in MC4R PVN neurons develop late onset obesity. **A-B.** Growth curves of A (male) and B (female) controls (Kcnj13^{+/+};MC4R^{+/+}, Kcnj13^{fl/fl}, Kcnj13^{+/+};MC4R^{Cre}) versus Kcnj13 Δ MC4R^{Cre} mice. **C-D.** Snout to anus length linear growth of C (male) and D (female) mice from groups shown in A-B measured at age 30 weeks. 6-25 mice per group, **P<0.005, ****P<0.0001. Data adapted from Anderson et al 2018, Journal of Neuroendocrinology. Reproduced with permission.

Though a new MC4R pathway involving Kir7.1 was identified, this ion channel has not been extensively profiled. Kir7.1 was first identified as a small unitary conductance Kir channel in 1998 with 37% identity to other inward rectifiers (Doring, Derst et al. 1998). Kir7.1 is highly expressed in the brain, specifically the choroid plexus. Expression in the PVN was confirmed by dual fluorescence *in situ* hybridization, though very low levels were found to be present (Ghamari-Langroudi, Digby et al. 2015). Roles for Kir7.1 have been demonstrated in the kidney and uterus,

with the retinal pigment epithelium being the area of greatest study (Suzuki, Yasuoka et al. 2003, McCloskey, Rada et al. 2014, Zhong, Chen et al. 2015). In the eye, Kir7.1 helps to maintain potassium homeostasis working in concert with the NaKATPase pump in the subretinal space (Kumar and Pattnaik 2014). Mutations within this ion channel lead to channelopathies such as Snowflake Vitreoretinal Degeneration (SVD) that result in various forms of blindness (see Chapter IV). Kir7.1 also supports potassium excretion in the Kidney where it is expressed in the medulla but not the cortex (Cornejo, Villanueva et al. 2018). While Kir7.1 is expressed in the trachea and testis, its function there has not been widely studied.

Research objective

With a physiological role for the MC4R-Kir7.1 interaction identified, determining the molecular mechanism of how this interaction occurs remains of keen interest. With a goal to develop therapeutics to treat disorders of energy homeostasis, understanding how these two proteins interact is of great importance. MC4R and Kir7.1 have also been shown to associate via co-immunoprecipitation as a complex in HEK293T cells (Ghamari-Langroudi, Digby et al. 2015) (See Chapter III, Fig 3.8). The functional coupling observed in the slice has also been reproduced in vitro using a fluorescence based assay (Ghamari-Langroudi, Digby et al. 2015, Litt, Cone et al. 2017). Given that MC4R can show signal bias with different ligands, one goal is to identify MC4R-Kir7.1 biased ligands in an attempt to avoid the pressor side effect. In our efforts to develop screening assays for these compounds, we discovered that other GPCRs may also associate with Kir7.1. The goal of this work, was to determine how Kir7.1 is regulated at a molecular level by protein-protein interactions with GPCRs. We discovered that channel glycosylation plays an essential role in Kir7.1 function, and that GPCRs can differentially regulate its degree of

glycosylation (Carrington, Hernandez et al. 2018). With Kir7.1 having different physiological roles across tissues, it is increasingly important to understand how this ion channel is controlled at the molecular level. This dissertation describes these new mechanisms of Kir7.1 regulation.

CHAPTER II

GLYCOSYLATION REGULATES FUNCTION OF INWARD RECTIFIER CHANNEL KIR7.1

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“G protein–coupled receptors differentially regulate glycosylation and activity of the inwardly rectifying potassium channel Kir7.1”

Abstract

Kir7.1 is an inwardly rectifying potassium channel with important roles in the regulation of membrane potential in retinal pigment epithelium, uterine smooth muscle, and hypothalamic neurons. Potassium channels undergo post translational modifications which can either promote or suppress function. Like other inward rectifiers, Kir7.1 was found to be glycosylated with both core and complex glycosylation. The only active glycosylation site of Kir7.1 was identified as N95, and a glycosylation resistant mutant of the channel N95Q was tested for an effect on function. Glycosylation was found to be essential for channel function, with the N95Q mutant displaying lower current amplitudes and reduced open states in single channel recordings. Mutation of a shared canonical Golgi export site present in Kir7.1 also led to a loss of complex glycosylation. Additionally, we found that the L241P mutation of Kir7.1 associated with Lebers congenital amaurosis (LCA), an inherited retinal degenerative disease, has significantly reduced complex

glycosylation. These results support the proposal that Kir7.1 requires glycosylation for channel function in tissues where it is expressed.

Introduction

Kir7.1, encoded by the *Kcnj13* gene, is a two-transmembrane domain potassium channel, closest in homology to Kir channels associated with potassium transport such as Kir1.1, 4.x, and 5.1 (Hibino, Inanobe et al. 2010). In comparison to other channels, Kir7.1 exhibits a small unitary conductance, and low dependence on external potassium (Doring, Derst et al. 1998). This was shown to be due to the presence of a methionine at position 125 in the pore, where other Kir channels have an arginine. Mutation of this residue to arginine was found to mimic/restore the conductance and potassium dependence observed in like-family channels (Doring, Derst et al. 1998). Kir7.1 is widely expressed, but particularly high expression has been reported in the retinal pigment epithelium (RPE), thyroid, uterine smooth muscle, small intestine and choroid plexus of the brain (Nakamura, Suzuki et al. 1999, Yang, Pan et al. 2003).

Until recently, Kir7.1 was primarily studied within the RPE. In RPE cells, Kir7.1 is found in the apical membrane close to photoreceptor neurons, where it is thought to contribute to ion homeostasis (Pattnaik, Tokarz et al. 2013). Mutations in Kir7.1 lead to snowflake vitreoretinal degeneration (SVD) and Leber congenital amaurosis (LCA), which are both retinal dystrophies (Hejtmancik, Jiao et al. 2008, Sergouniotis, Davidson et al. 2011, Zhang, Zhang et al. 2013, Khan, Bergmann et al. 2014, Pattnaik, Shahi et al. 2015). In *jaguar/obelix* zebrafish, mutations in Kir7.1 produces spotted pigment patterns rather than stripes, and also results in a failure of melanosomes to respond to changes in light (Iwashita, Watanabe et al. 2006). These functional mutations of

Kir7.1 in RPE cells and melanocytes have been characterized and shown to cause alterations in channel currents resulting in the described pathologies.

Glycosylation of proteins has been widely studied and can have various effects on protein function from supporting protein folding, to ensuring surface expression. Glycosylation begins co-translationally in the ER where the first glycan is added, and is completed in the Golgi complex with the addition of complex glycans. In the case of N-linked glycosylation, the first core glycan is added by a complex of enzymes to an accessible asparagine residue with the protein sequence Asn-X-Ser/Thr (Aebi 2013). ER added glycans are then pruned in the Golgi before the addition of more complex structures. Proteins with ER only glycans are referred to as immature or core-glycosylated, and those with more complex Golgi added structures are termed mature or complex glycans. Glycans added to the protein in the Golgi are resistant to the endoglycosidase Endo H, but core glycans are sensitive. This can be exploited to probe the protein trafficking and maturation of glycosylated proteins.

A role for glycosylation in Kir channels has not been widely studied with only a few channels reported to have glycosylation. Kir1.1 remains the only channel for which glycosylation was found to have a role in function, with a glycosylation mutant tested having reduced channel open probability (Schwalbe, Wang et al. 1995). While other channels such as Kir3.x and Kir4.1 are glycosylated, glycans are either not essential for function or a role in function has not been tested. Among inward rectifiers, a role for Kir7.1 has been mainly studied in the RPE, and how variants of the channel affect function and cause retinopathies. On the molecular level, it remains unclear how certain posttranslational modifications of Kir7.1 may affect channel function. While phosphorylation of the channel has been previously described, glycosylation of Kir7.1 and its role

in channel function has yet to be determined (Zhang, Zitron et al. 2008). Here, we describe characterization of the sole glycosylation site in Kir7.1.

Results

Kir7.1 undergoes complex glycosylation

To overexpress 3xFLAG tagged Kir7.1 in mammalian cells, we used a construct of the human cDNA sequence inserted into a pciNeo plasmid. Transient expression of the channel in WT HEK 293T cells led to three predominant bands observed, with the lowest band migrating as a doublet (Fig. 2.1). Using endoglycosidases, we confirmed the upper 50kDa band as the Endo H resistant mature/complex glycosylated form of the channel, indicating that these bands are Kir7.1 that include glycans added in the Golgi complex. With Endo H treatment, the 37kDa doublet shifted to a single band, indicating Endo H sensitivity. As Endo H sensitive glycans are added in the endoplasmic reticulum, we identified this upper 37kDA band as the core glycosylated form, and classified this lower band as the unglycosylated form of Kir7.1. Additionally, treatment with the glycosidase PNGase F, which cleaves all asparagine linked (N) glycans of proteins, confirmed that the single migrating band at 37kDa is the unglycosylated protein, and that Kir7.1 glycosylation is N-linked.

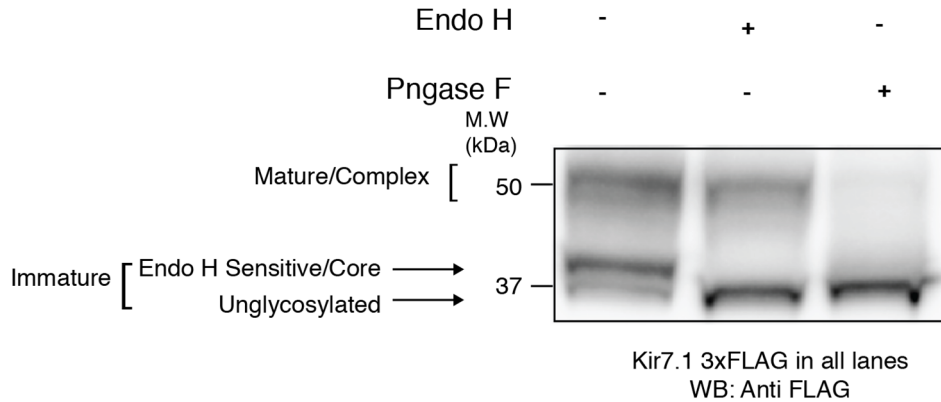


Figure 2.1 Inhibition of complex glycosylation of Kir7.1 by the β 2AR.

Western blot analysis of Kir7.1 whole cell lysates untreated, or treated with Endo H or PNGase F. Enzymatic treatment identified the 50KD Endo H resistant doublet as the complex or mature glycosylated form (Lane 2). The 37KD band was identified as the core glycan form due to endo H sensitivity (lane 2), and the lowest band in lane 3 as the un-glycosylated PNGase F cleaved form. Representative of three independent experiments.

Lack of glycosylation of Kir7.1 alters channel gating

Within the Kir family, the functional role of glycosylation has been studied for ROMK (Kir1.1) and GIRK1 (Kir3.1) (Pabon, Chan et al. 2000). While loss of glycosylation at Asn119 in GIRK1 was reported not to be required for function, loss of glycosylation at Asn117 in ROMK1 reduced open channel probability, and whole cell currents (Schwalbe, Wang et al. 1995). Additionally, though Kir3.4 is reported to have an asparagine accessible for glycosylation, the site was found to be inactive (Pabon, Chan et al. 2000). Thus, divergent roles for glycosylation exist within the family. Based on these studies, we aligned the Kir7.1 protein sequence with ROMK and GIRK1 and determined that Kir7.1 also shared a highly conserved glycosylation site (Fig. 2.2A). Kir7.1 also shared the canonical sequence associated with N-linked glycosylation- N-X-T/S, where Kir7.1 has a Threonine in the third position. We mutated this site to a glutamine (Kir7.1-N95Q) and confirmed by western blot analysis the loss of both core and complex forms of glycosylation

(Fig. 2.2B). Furthermore, we generated a model of Kir7.1 based on the Kir2.2 crystal structure using SWISS_MODEL, which revealed the likely glycosylation site to be in the outer loops gating the pore (Fig. 2.2C). This was suggestive of a functional role for glycosylation in Kir7.1. To assess this, we performed single channel recordings using the M125R mutant of Kir7.1, to compare the WT fully glycosylated channel (Kir7.1-M125R), to the un-glycosylated mutant channel (Kir7.1-M125-N95Q).

Wild type Kir7.1 displayed clusters of openings with a main amplitude level of ~ 1 pA (Fig. 2.3A, 3D). Lack of glycosylation at the N95 position of Kir7.1 channels caused openings to occur at an amplitude level of ~ 0.6 pA (N95Q = 0.65 ± 0.03 pA, n=6; wt = 1.11 ± 0.07 pA, n=7. $P=0.0001$) with a reduced open probability (N95Q = 0.23 ± 0.05 , n=6; wt = 0.41 ± 0.06 , n=7. $P=0.0484$) (Fig. 2.3B, 2.3E). Both channels opened to at least three open states ($O1$, $O2$ and $O3$), with no changes in the relative occurrence of opening events (N95Q = 9.09 ± 3.8 ms, n=6; wt = 17.9 ± 3.5 ms, n=7. $p=0.1188$). Thus, the lack of glycosylation leads to a clear reduction in function of individual Kir7.1 channels.

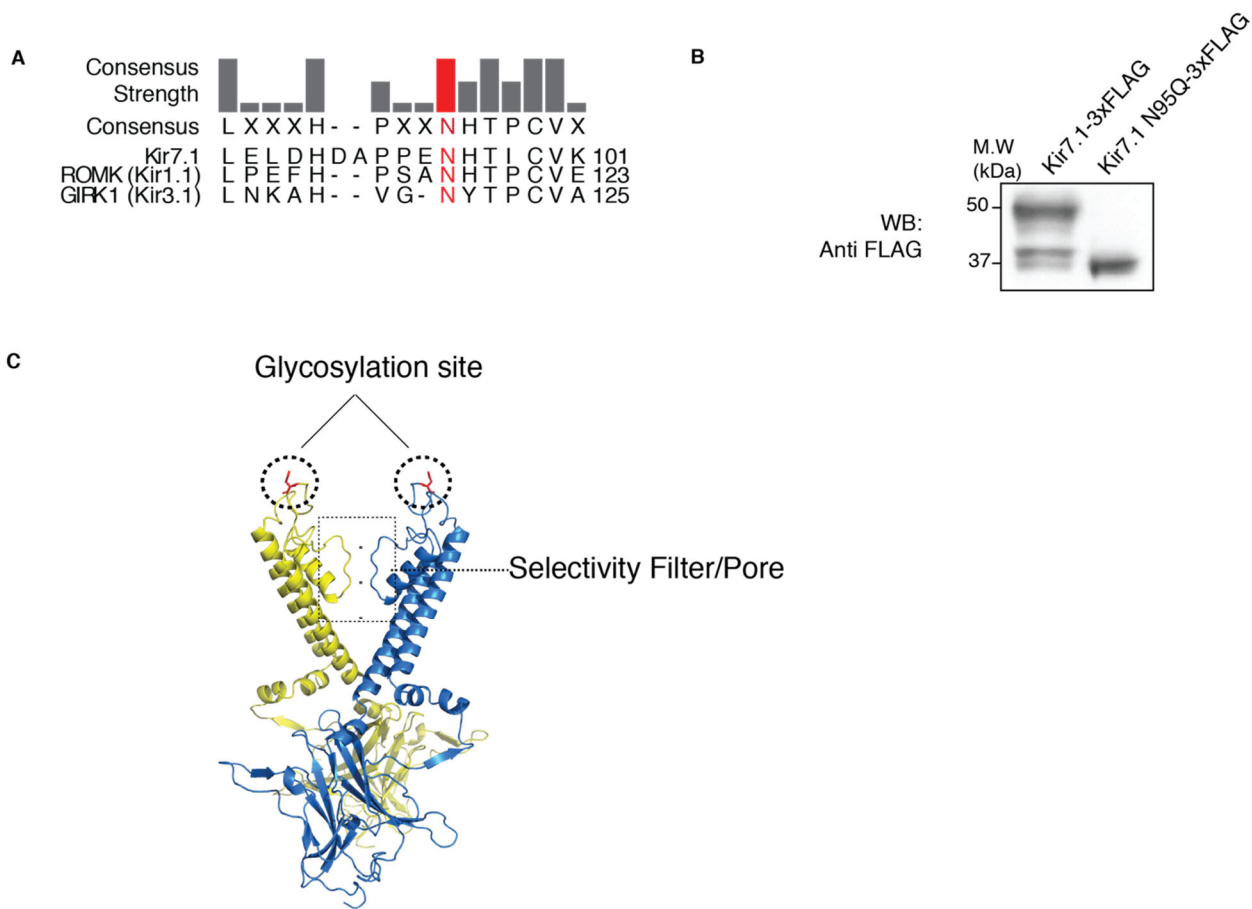


Figure 2.2 Creation of an unglycosylated mutant of Kir7.1.

A. Lasergene MegAlign software was used to confirm Kir7.1 conserved glycosylation site of N95 compared to other known glycosylated Kir channels, ROMK(Kir1.1) N117 and GIRK1 N119 (Pabon, Chan et al. 2000). This site also has a known N-linked glycosylation sequence (N-X-T/S) (Helenius and Aebi 2004). **B.** The N95 site of Kir7.1 was mutated to glutamine N95Q, and the whole cell lysates of the 3xFLAG tagged N95Q mutant and the similarly tagged WT channel were analyzed by western blot analysis using an anti-flag antibody. No higher migrating forms of Kir7.1 were observed in the N95Q mutant. **C.** Homology modeling of Kir7.1 based on Kir2.2 (PDB 5kuk) revealed the location of the N95 site in the outer loops which gate the channel pore. This suggested a possible role for glycosylation in channel function. Modeling performed using SWISS-MODEL, and image generated with PyMOL. Transfection levels were as in Figure 2.1.

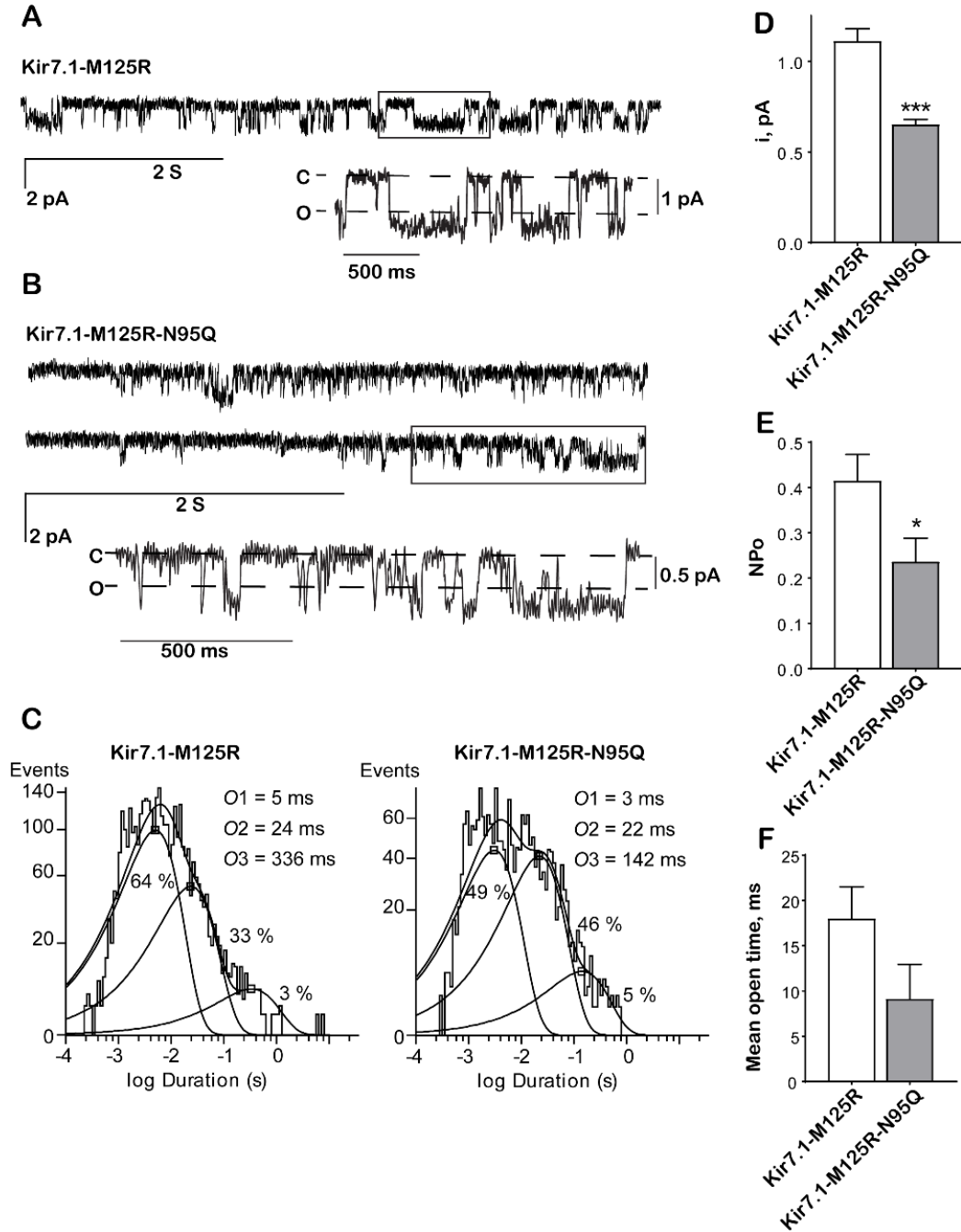


Fig.2.3 Representative single-channel current traces from cell-attached patches of Kir7.1 Kir7.1-M125R (**A**) and Kir7.1-M125R^{N95Q} (**B**) channels recorded from HEK293T cells. Patches were voltage clamped at 130 mV. Openings are downward and each representative trace was a continuous 5000-ms recording. (**C**) Average time histograms of openings (*O1*, *O2* and *O3*) events are shown with the time constants (ms) and the relative contribution (%) of open events. Bar graphs summarize the effects of Kir7.1-M125R^{N95Q} on amplitude (**D**), mean open probability (NPo) (**E**), and mean open time (**F**) of channels. Values are expressed as mean \pm S.E.M (WT, N = 6; NQ, N = 7). Statistical differences were determined using unpaired t-test relative to wt. C and O refer to

the closed and open states, respectively. Wt, wild type. NQ, N95Q. Experiment performed by Ciria C. Hernandez

Kir7.1 has a conserved Golgi export site, essential for complex glycosylation

With recent reports of a Golgi export site required for surface expression of Kir channels (Ma, Taneja et al. 2011, Li, Ortega et al. 2016), we found that Kir7.1 shared a similar site to the N and C terminal regions of Kir2.1, 4.1, and 5.1 that has been identified as essential for Golgi export (Fig. 2.4A-B) (Li, Ortega et al. 2016). Within the channels mentioned, deletion of the serine and tyrosine (SY) residues within the C-terminal site was found to be sufficient to prevent Kir export from the cis-Golgi and trans-Golgi compartments of the cell, where complex glycosylation takes place (Ma, Taneja et al. 2011, Li, Ortega et al. 2016). Additionally, while WT Kir2.1 is not glycosylated, it was reported that deletion of SY in Kir2.1 also reduced complex glycosylation of an artificially placed asparagine residue (Ma, Taneja et al. 2011). Given the apparent conservation of this site in Kir7.1, we tested whether deletion of these residues would alter WT Kir7.1 glycosylation. Deletion of the SY motif in Kir7.1 led to a significant loss of complex glycosylation with a ratio of <0.1 in Cos1 cells (Fig. 2.4 C-D). We established a metric for the proportion of Kir7.1 complex glycosylation, by determining the ratio of mature/complex glycosylation, to immature core glycosylated plus unglycosylated forms of the protein. Cos1 cells were used, as published studies of the export site in other Kir channels were performed in Cos cell-lines (Grieve and Rabouille 2011). We further confirmed the loss of complex glycosylation by endoglycosidase digestion using Endo H and PNGase F (Fig. 2.4E). This indicates that the Golgi export residues SY in Kir7.1 are essential for complex glycosylation of Kir7.1. While the SY mutant did have a significantly low glycosylation ratio, we also observed a glycosylation ratio of <0.8 in the WT

channel in Cos1 cells (Fig. 2.4E), lower than the typical ratio of 1 observed in HEK293T cells (see Fig.1.2 chapter III).

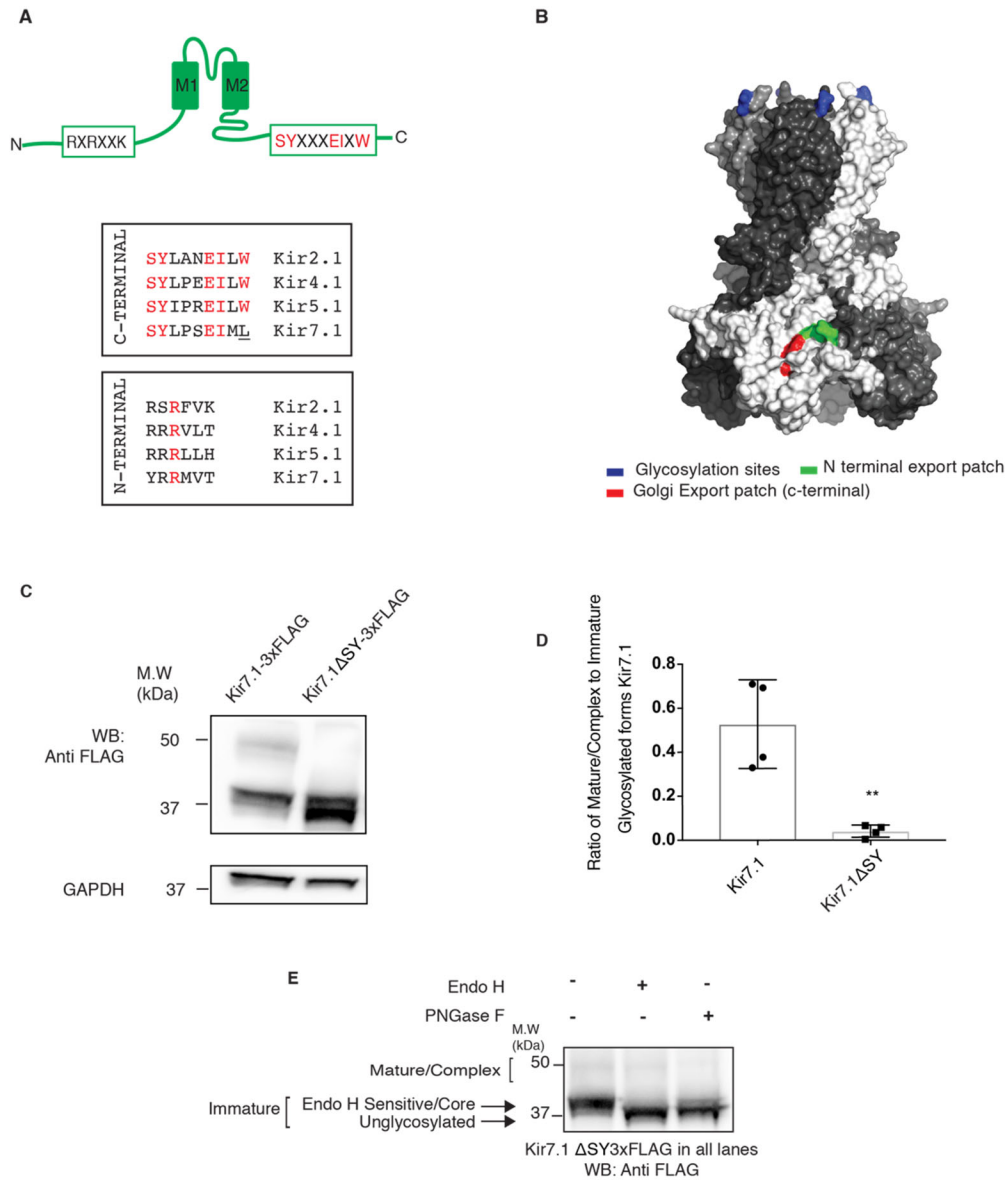


Figure 2.4 A conserved Golgi transport signal is required for complex glycosylation of Kir7.1. **A.** Schematic showing a two pore domain Kir channel subunit with two transmembrane regions M1 and M2. The consensus N terminal and C terminal Golgi export patch residues are shown, with known essential residues shown in red. Clustal alignment of the N and C terminal regions of the Golgi export patch of other closely related Kir channels which have been previously identified (Li, Ortega et al. 2016). Kir7.1 shares a highly conserved patch, except for a C terminal leucine residue where other channels have a tryptophan. **B.** SWISS-Model of Kir7.1 structure based on

the crystal structure of Kir2.2 (PDB5KUK). Blue highlighted regions show the glycosylation site in each monomer of the channel. The N and C terminal portions of the Golgi export patch are highlighted in green and red respectively. **C.** Deletion of the serine and tyrosine residues in the Golgi export patch of Kir7.1 led to a loss of complex glycosylation compared to the WT channel determined by western blot analysis in Cos1 cells. **D.** Compared to the WT channel, essentially no complex glycans were observed indicated by the significantly low ratio of 0.1 $p < 0.005$, (unpaired t-test). **E.** Enzymatic digestion of whole cell lysates of the WT versus Kir7.1 SY Δ mutant from HEK293T cells confirmed only the immature forms of Kir7.1 are expressed. Data analyzed from four independent experiments analyzed in Graphpad Prism, with one-way ANOVA. Error bars for all data represents S.E.M.

Kir7.1 forms heterotetramers of immature, unglycosylated, and fully glycosylated subunits

Having established that complex glycosylation of Kir7.1 is required for function, we tested whether the channel exists as homo- or hetero-tetramers of subunits with no glycosylation, core glycosylation, and complex glycosylation. We co-expressed flag tagged Kir7.1-N95Q or Kir7.1 Δ SY with an HA tagged WT Kir7.1 channel. HA tagged WT Kir7.1 co-immunoprecipitated with both the un-glycosylated (N95Q) and immature glycosylated (Δ SY) forms of the channel, in addition to the fully glycosylated WT control (Fig. 2.5). This suggests that non-functional Kir7.1 subunits may form tetramers with WT subunits, and thus may reduce channel function through “subunit poisoning”. Theoretically, a threshold may exist where a minimum number of subunits must be fully glycosylated in order for the channel to be functional. Thus, the presence of an unglycosylated subunit may indeed poison the entire tetramer functionally, similar to a dominant negative mutant. This however has not been definitively tested.

Retinopathy associated mutations of Kir7.1 also alter channel glycosylation

Genetic studies have linked retinopathies of the eye to mutations in Kir7.1, demonstrating that Kir7.1 plays a key role in the function of the retinal pigment epithelium. Snowflake Vitreoretinal Degeneration (SVD) and Lebers Congenital Amaurosis (LCA) are two Kir7.1

associated hereditary eye disorders, where known variants of Kir7.1 lead to loss of channel function (Kumar and Pattnaik 2014). We wanted to determine if glycosylation is also affected by any of these disease mutations, and a few known variants of Kir7.1 namely R162W, R162Q, Q117R, and L241P were tested. These are all considered to be loss of function mutations, except R162Q which is linked to blindness, but has no reported phenotype (Kumar and Pattnaik 2014). Notably, none of these mutations are in known sites required for complex glycosylation. Interestingly, we observed a significant reduction in glycosylation in the L241P mutation (Fig 2.6), in addition to reduced levels of protein expression.

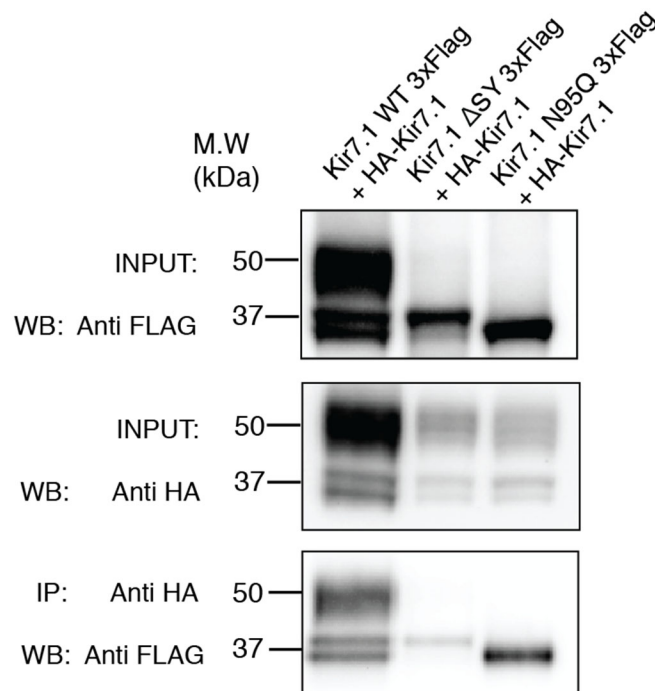


Figure 2.5 Kir7.1 forms heterotetramers of immature and unglycosylated subunits. HEK293T cells were transiently transfected with HA-Kir7.1 with either flag tagged Kir7.1 N95Q, Kir7.1 Δ SY or Kir7.1 WT as a control; transfection levels were as in Fig. 2.1. Cell lysates were immunoprecipitated with an HA antibody and immunoblotted with anti-flag. Blot is representative of three independent experiments. See methods for additional experimental details.

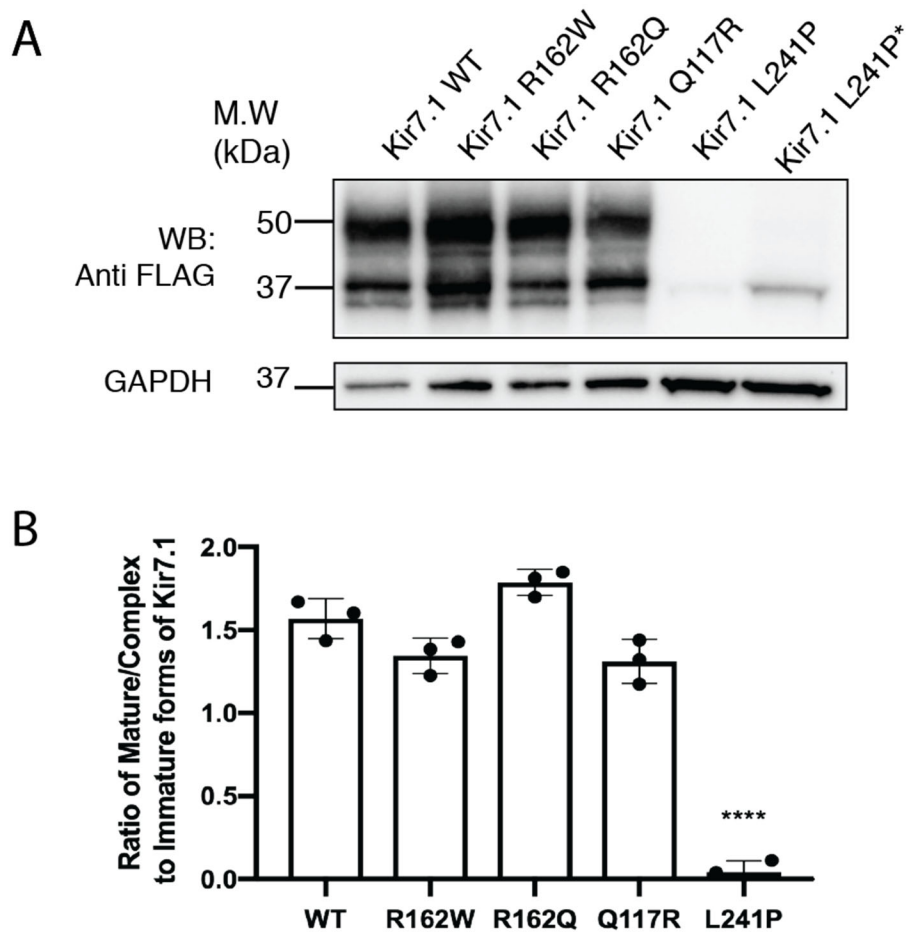


Figure 2.6. A retinopathy associated variant of Kir7.1 has altered complex glycosylation. **A.** Western blot analysis of three known variants of Kir7.1, following transfection of Kir7.1 mutants indicated; transfection levels were as in Fig. 2.1. Whole cell lysates of HEK293T cells expressing a known variant of Kir7.1 were analyzed for changes in glycosylation. Kir7.1 L241P in lane 5 not only had lower expression, but the absence of complex glycosylation. *15ug of protein were loaded versus 7.5ug in all other lanes. Blot is representative of four independent experiments. **B.** Densitometric analysis was performed to obtain the ratio of mature to immature glycosylation. Data representative of three independent experiments. $P < 0.0001$ with one-way ANOVA. Error bars for all data represent S.D..

Discussion

Glycosylation has been previously reported for two members of the same family of channels as Kir7.1, Kir1.1 (ROMK) and Kir3.1 (Pabon, Chan et al. 2000). Loss of glycosylation in Kir1.1 was shown to decrease whole cell currents due to a significant reduction in open channel probability (Schwalbe, Wang et al. 1995). We observed similar results in our N95Q glycosylation resistant mutant by single channel recordings. Interestingly, Kir3.1, though having an active glycosylation site, is not reported to require glycosylation for function. While Kir3.1 and Kir3.4 form complexes, Kir3.4 has a consensus glycosylation site that is reported to be inactive (Pabon, Chan et al. 2000). Thus, within the two-transmembrane domain family of Kir channels, a divergent role for channel glycosylation exists. Additionally, the Kir4.1 channel has been shown to be differentially glycosylated in different tissues. While higher amounts of complex glycosylation of Kir4.1 have been observed in kidney versus brain lysates, a functional role for glycosylation has not been investigated (Hibino, Fujita et al. 2004).

Kir1.1 and ROMK have the highest similarity to inward rectifier to Kir7.1, with ~38% sequence homology. As mentioned previously, Kir1.1 is the only inward rectifier with a clear reported role for glycosylation. The N95 site in Kir7.1 appears structurally related to the N117 site in Kir1.1 which suggested a similar role for glycosylation in Kir7.1, as validated here. From sequence analysis, the glycosylation site of both these channels is present in the turret region of the channel, an extracellular loop before the pore helix between the two transmembrane domains (Shealy, Murphy et al. 2003). This region has high variability across the large potassium channel family, with a potential function for sensitivity to toxin binding (Shealy, Murphy et al. 2003). Based on our homology model of Kir7.1 (Fig. 2.2C), these extracellular loops would house the

relatively large glycans at N95, that by our estimate from western blot analysis are over 13kDa in size. However, the exact mechanism by which the presence of these glycans is essential for a fully functional channel is not clear. Structural comparison of the Kir7.1 model to the Kir2.2 crystal structure also shows that the turret region of Kir7.1 is longer than that in Kir2.2 with additional prolines present in the loop. These proline residues may introduce additional turns in the loop either towards or away from the pore. However, we were unable to predict these conformations with SWISS-MODEL. A crystal structure of this extracellular region of Kir7.1 would indeed allow us to develop an hypothesis for the mechanism by which glycosylation aids channel function.

It was interesting to observe that the L241P LCA associated variant of Kir7.1 exhibited a near complete loss of complex glycosylation. This mutation has not been extensively studied, but the loss of channel function was predicted due to its association with LCA, a severe form of retinal degeneration (Zhang, Zhang et al. 2013). This mutation is thought to be in a β sheet of the protein, near the C-terminal region, and is predicted to affect protein folding. It remains unknown whether the change in structure is responsible for the loss of glycosylation and thus a loss of function, or a reduction in protein stability, or loss of activity due to another structural defect (Sergouniotis, Davidson et al. 2011). The protein did not express well following transfection of HEK293 cells (Fig. 2.6), but data are not available to assess whether or not the mutation reduces expression levels in RPE cells in vivo. Both the WT and L241P Kir7.1 proteins have now been purified by Dr. Alys Peisley in the Cone laboratory, and their structures are being determined by Cryo-EM.

We have observed that deletion of the serine and tyrosine residues within the Golgi export site of Kir7.1 also inhibits complex glycosylation, but leaves core glycosylation intact. The SY mutant of Kir7.1 described here may thus be a useful tool in future experiments to determine if core glycosylation is sufficient for maintenance of normal channel function. Complex

glycosylation is reported to occur at different stages during passage from the cis-medial-trans Golgi before export to the surface (Stanley 2011). Within other Kir channels, the Golgi export site has been identified as a binding site of the AP-1 gamma adaptin protein which is required for export to the cell surface (Ma, Taneja et al. 2011, Li, Ortega et al. 2016). In Kir2.1, deletion of these two residues reduced complex glycosylation of an artificial site placed within the channel by mutagenesis, with an intermediate glycosylation pattern observed (Ma, Taneja et al. 2011). They argue that as complex glycosylation occurs in the trans-Golgi network, loss of the export site traps Kir2.1 in both the cis and trans Golgi network, preventing further transport leading to incomplete complex glycosylation. However, in Li et al 2016, the authors comment that in comparison to other Kir channels, Kir7.1 is the only channel that does not seem to share the full export site. While we have confirmed the export site to be essential for complex glycosylation, these two residues do not appear to be required for Golgi export (data not shown), however, this was expected as we did see immature forms of the WT channel at the surface in biotinylation experiments described in chapter III.

Through sequence analysis and site directed mutagenesis we have shown that Kir7.1 requires glycosylation for full channel function. Few antibodies exist that allow the detection of glycosylated forms of Kir7.1. However, as these studies were being completed a new HA-Kir7.1 Crispr mouse model described by Cornejo et al 2018 was demonstrated to express highly variable levels of Kir7.1 complex glycosylation in different tissues. Thus, this animal model may be useful to study the physiological functions of Kir7.1 glycosylation in vivo.

CHAPTER III

GPCRS DIFFERENTIALLY ALTER THE COMPLEX GLYCOSYLATION OF KIR7.1

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“G protein–coupled receptors differentially regulate glycosylation and activity of the inwardly rectifying potassium channel Kir7.1”

Abstract

Kir7.1 is an inwardly rectifying potassium channel which plays varying roles in potassium homeostasis. Recently, new roles are being discovered such as Kir7.1 coupling to the melanocortin-4 receptor (MC4R), a G protein–coupled receptor (GPCR) in hypothalamic neurons. Regulation of G protein–coupled inwardly rectifying potassium (GIRK) channels by GPCRs via the G protein $\beta\gamma$ subunits has been well characterized. However, how a non-GIRK channel like Kir7.1 is regulated by GPCRs is not understood. We report here that Kir7.1 is regulated by GPCRs through a different mechanism. We have previously established that glycosylation is essential for Kir7.1 channel function. Using Western blot analysis, we observed that multiple GPCRs tested caused a striking reduction in the complex glycosylation of Kir7.1. Further, GPCR-mediated reduction of Kir7.1 glycosylation in HEK293T cells did not alter its expression at the cell surface but decreased channel activity. The MC4R, a GPCR previously reported to induce ligand-regulated activity of this channel, is the only GPCR tested that does not have this effect on Kir7.1. These

results indeed reveal a new mode of Kir7.1 channel regulation through GPCR mediated suppression of glycosylation.

Introduction

Among inward rectifiers, Kir7.1 is one of the least studied ion channels. While its role in regulating potassium homeostasis in the RPE is well understood, its function and regulation in other tissues is unclear (Kumar and Pattnaik 2014). Recent studies have identified a role for Kir7.1 in the regulation of uterine smooth muscle excitability (McCloskey, Rada et al. 2014). Specifically, Kir7.1 expression is elevated by 30-fold mid-gestation, hyperpolarizing muscle cells and reducing uterine excitability. This expression falls off toward the end of pregnancy, leading to parturition. In the mouse, Kir7.1 null mutations cause perinatal lethality, and this was determined to be due to a role for Kir7.1 in tracheal tubulogenesis (Yin, Kim et al. 2018). Kir7.1 was also revealed to be a potential regulator of neuronal excitability in the paraventricular nucleus of the hypothalamus, through modulation of channel activity by the G-protein coupled MC4R (Ghamari-Langroudi, Digby et al. 2015). Activation of MC4R led to robust neuronal depolarization, which was found to be mediated by Kir7.1 channel closure. Conversely, inhibition of MC4R by an endogenous inverse agonist, AgRP, led to channel opening and hyperpolarization of the neuron. These results are interesting in that Kir7.1 is not a canonical G-protein coupled inwardly rectifying (GIRK) channel such as Kir3.x. Furthermore, in transfected HEK293T cells, Kir7.1 was found to exist in a complex with MC4R (Ghamari-Langroudi, Digby et al. 2015).

Mechanisms by which non-GIRK channels interact with GPCRs vary, with some channels behaving like ‘GIRKs’, such as Kir2.x channels. Specifically, unlike Kir2.1, it has been reported

that Kir2.3 channels can be inhibited directly by $G_{\beta\gamma}$ complexes (Cohen, Sha et al. 1996). Moreover, within dendritic spines of cholinergic neurons, M1 receptor activation has been shown to lead to inhibition of Kir2.x channels through G_q activation of phospholipase C (PLC), which depletes the second messenger phosphatidylinositol 4,5-bisphosphate (PIP₂) that is needed for Kir channel function. (Shen, Tian et al. 2007). Kir7.1 likewise has a PIP₂ dependence for activity, and was recently shown in RPE cells to be regulated by the G_q coupled Oxytocin GPCR, through PLC mediated inhibition (York, Halbach et al. 2017). However, the MC4R mediated closure of Kir7.1 appears to be independent of G-proteins (Ghamari-Langroudi, Digby et al. 2015). The mechanism by which this occurs is unknown, but examples exist of accessory proteins regulating Kir channels in the absence of G proteins and other second messengers. For example, Kir6.2 and the non-GPCR sulphonylurea receptor (SUR), a target of sulphonylurea drugs used to treat diabetes, form the K_{ATP} channel (Raphemot, Swale et al. 2014). This hetero-octamer composed of Kir6.2 subunits in 1:1 stoichiometry with SUR1/2 is expressed in the pancreas and ATP binding to the SUR subunits increases channel open probability (Aittoniemi, Fotinou et al. 2009). SUR communicates with Kir6.2 through direct interaction between the C terminal tail of Kir6.2 and an intracellular loop of SUR (Li, Wu et al. 2017, Martin, Yoshioka et al. 2017).

In our initial investigations of potential molecular mechanisms of interaction between Kir7.1 and MC4R, we observed that co-expression of several GPCRs with Kir7.1 in HEK293T cells led to a dramatic reduction in complex glycosylated forms of Kir7.1 present in the cell. A review of the role of glycosylation within this family of inwardly rectifying potassium channels revealed that glycosylation of Kir1.1, a close family member, reduces open channel probability (Schwalbe, Wang et al. 1995, Pabon, Chan et al. 2000). We have also previously described, that Kir7.1 likewise requires glycosylation for channel function (see Chapter II). In patients with

multiple sclerosis, autoantibodies against glycosylated forms of Kir4.1 have been reported but a role for this in the pathophysiology of the disease is unclear (Pröbstel, Kuhle et al. 2016, Marnetto, Valentino et al. 2017). We report here on a novel functional role of GPCRs in the complex glycosylation and function of Kir7.1.

Results

Kir7.1 channel glycosylation is altered by the beta-2-adrenergic receptor (β 2AR)

The alpha-MSH neuropeptide regulates food intake and energy expenditure through the MC4R, a G protein-coupled receptor. Studies of MC4R neurons suggested this receptor regulates neuronal depolarization and activation by ligand-mediated regulation of the conductance of Kir7.1 (Ghamari-Langroudi, Digby et al. 2015). Further, these studies supported a G protein-independent mechanism of regulation of Kir7.1. To investigate this unusual mode of Kir regulation, we first co-expressed a c-terminal flag tagged Kir7.1 with either 3HA-MC4R, or as a control with the widely studied beta-2-adrenergic receptor (3HA- β 2AR) in HEK293T cells in the absence of ligand. We selected the β 2AR as a control as this receptor also couples to $G\alpha_S$. Unexpectedly, we observed a significant reduction in the 50kDa migrating doublet of Kir7.1 when co-expressed with β 2AR, whereas MC4R caused a small increase in this form of the channel in some experiments (Fig. 3.1A). Treatment of cells with melanocortin agonists, shown to reduce Kir7.1 currents in PVN MC4R neurons (Ghamari-Langroudi, Digby et al. 2015), had no effect on the typical amount or pattern of Kir7.1 bands observed (not shown). Using endoglycosidases, we previously confirmed the upper 50kDa bands as the Endo H resistant mature/complex glycosylated form of the channel, indicating that these bands are Kir7.1 that include glycans added in the Golgi complex

(Chapter II Fig 2.1). We established a metric for the proportion of Kir7.1 complex glycosylation, by determining the ratio of mature/complex glycosylation, to immature core glycosylated plus unglycosylated forms of the protein. While some variability in this ratio was seen, a value of approximately 1 was typically observed across multiple Kir7.1 expression trials in HEK293T cells in the absence of GPCR co-expression. Co-expression with β 2AR reduced the ratio of glycosylation to less than 0.5, whereas MC4R exhibited a trend towards increasing this ratio, indicating opposing effects of these GPCRs (Fig. 3.1B). As differential glycosylation of Kir channels have been reported across different tissue subtypes (Hibino, Fujita et al. 2004), we tested whether β 2AR would likewise mediate a loss of glycosylation in the Cos1 cell line. The striking loss of Kir7.1 glycosylation observed within HEK293T cells when co-expressed with β 2AR was repeated within Cos1 cells compared to the WT channel, and co-expression with MC4R again lacked the same effect (Fig 3.1C-D). This further indicated the strong specificity of the β 2AR-Kir7.1 interaction.

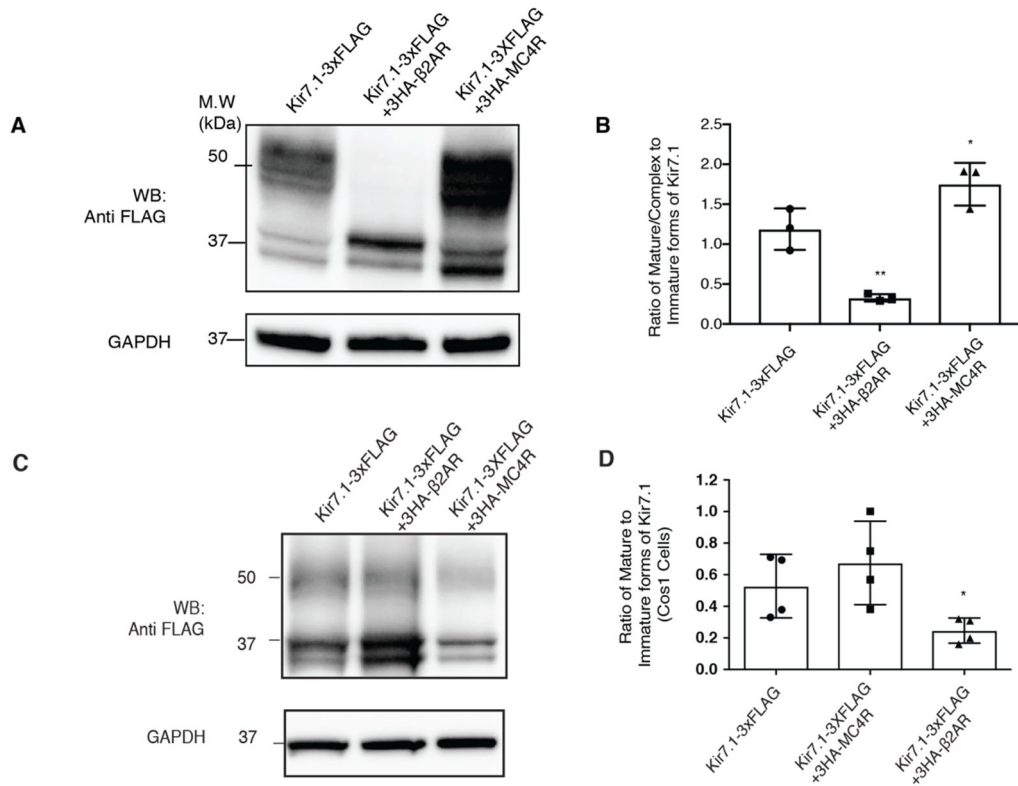


Figure 3.1 Inhibition of complex glycosylation of Kir7.1 by the β 2AR.

A. Western blot analysis from whole cell lysates of transiently transfected HEK293T cells with 3XFLAG tagged Kir7.1 alone, or co-expressed with either 3HA tagged β 2AR or the MC4R. Transfections in A and B were performed using 0.25ug of expression vector for the channel and the β 2AR. 2.5 ug of expression vector for the MC4R was used to achieve receptor expression levels comparable to that seen with the β 2AR (see Fig. 3.2) **B.** Densitometric quantification of the blot shown in A, showed that MC4R had a modest increase in the ratio of mature to immature forms of kir7.1 $p < 0.05$, whereas β 2AR significantly reduced the ratio of mature forms of Kir7.1 $p < 0.005$. **C.** β 2AR also reduces the ratio of mature to immature glycosylated Kir7.1 in Cos1 cells. Western blot analysis of whole cell lysates from Cos1 cells co-expressing Kir7.1-3XFLAG with either 3HA- β 2AR or 3HA-MC4R. **D.** 3HA- β 2AR also reduced Kir7.1 mature glycosylation in Cos1 cells as shown in the quantification by densitometric analysis, $p < 0.05$. Data analyzed from three-four independent experiments analyzed in Graphpad Prism, with one-way ANOVA. Error bars for all data represents S.D.

Dose-responsive inhibition of Kir7.1 complex glycosylation with increased β 2AR expression

With the striking observation of reduced Kir7.1 glycosylation following β 2AR but not MC4R co-expression, we were curious whether differences in receptor expression levels could account for the proportion of Kir7.1 complex glycosylation observed. To examine this, we co-expressed Kir7.1 with increasing amounts of either MC4R or β 2AR protein and assessed glycosylation by western blot analysis. We observed that increasing the amount of MC4R expressed had no effect on proportion of Kir7.1 complex glycosylation, whereas increasing amounts of β 2AR potentiated the loss of complex glycosylation (Fig. 3.2A-B). Moreover, co-expression with β 2AR appeared to be increasing the amounts of core-glycosylated/immature Kir7.1 expressed. We determined the change in ratio of Kir7.1 glycosylation with changes in receptor expression, and β 2AR mediated a progressive reduction in the ratio of complex to immature forms (unglycosylated and core glycosylated) of Kir7.1 (Fig. 3.2C). Increases in the amount of MC4R protein expressed did not have an effect on the ratio of mature/immature forms of Kir7.1. As a control, and to determine whether the effect on glycosylation was specific to Kir7.1, we assessed whether overexpression of either GPCR would alter the glycosylation of the widely studied vesicular stomatitis virus glycoprotein (VSVG) tagged with GFP. VSVG-GFP is used to elucidate protein trafficking, where forms of the protein that have mature/complex glycosylation are Endo H resistant. This can be determined by observing a band shift when comparing Endo H treated versus untreated cell lysates of VSVG-GFP with either GPCR. We first treated cells transiently expressing VSVG-GFP only with a transport inhibitor (BFA/Monesin) to prevent complex glycosylation and observed the band shift in comparison to untreated samples as a control (Fig. 3.2D). Keeping expression levels of Kir7.1 constant throughout (.25ug of expression vector), and varying β 2AR expression five-fold (from 0.05 to 0.25ug of expression vector), the high

expression levels of β 2AR that altered Kir7.1 glycosylation in Fig. 3.1A (0.25ug of expression vector), had no effect on the glycosylation of the VSVG-GFP protein (Fig 3.2E, lanes 7-8). Only when β 2AR was overexpressed 3-fold relative to Kir7.1 (0.25ug of Kir7.1: 0.75 ug of β 2AR) was VSVG-GFP glycosylation affected, most likely due to squelching of glycosylation machinery. With the exception of the overexpression shown in Fig 3.2E, lanes 9-10, the western blot analyses reported here never exceeded the 1:1 transfection of 0.25ug each of Kir7.1 and β 2AR expression vectors. MC4R also did not alter VSVG-GFP glycosylation at levels used throughout this experiment (Fig. 3.2F). These data further indicated a unique effect of β 2AR expression, in contrast with MC4R, on suppression of Kir7.1 complex glycosylation, at expression levels that have no effect on the glycosylation of VSVG protein.

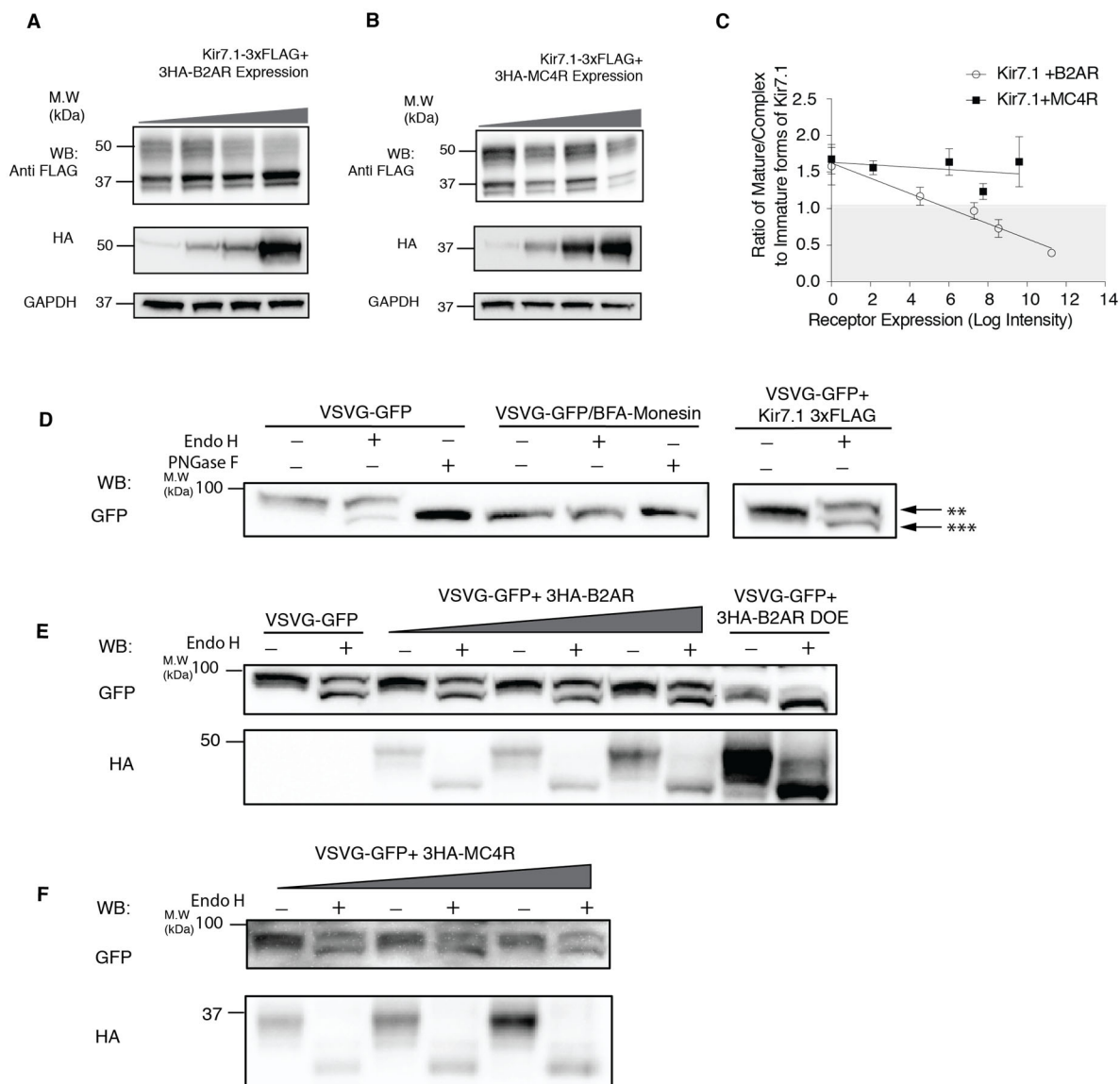


Figure 3.2 Increasing amounts of the β 2AR increase the amounts of unglycosylated and core-glycosylated forms of Kir7.1, while the melanocortin-4 receptor does not. **A-B** HEK293T cells were transiently transfected with a fixed amount of 3xFLAG tagged Kir7.1 (0.25ug) in the presence of increasing concentrations of 3HA- β 2AR (0.05, 0.08, 0.125, 0.25ug) or 3HA-MC4R plasmid DNA (0.5, 0.8, 1.25, 2.5ug). Whole cell lysates were analyzed by western blot analysis. To assess receptor expression levels, samples were rerun on a separate blot, and imaged with anti HA. This blot was stripped and reprobred for the loading control GAPDH to confirm plasmid concentration based increase in receptor protein levels. **C**. Densitometric analysis of the anti-FLAG blot to determine the ratio of mature to complex glycosylation. Densitometric analysis was also performed on the receptor blot, and normalized to the GAPDH loading control for both β 2AR expression and MC4R expression. The ratio of mature to immature Kir7.1 glycosylation was plot against Log2 receptor intensity values, and a linear regression line fitted to the data points. Data representative of at-least 3 experiments. **D-F**.

Comparable increases in receptor expression do not alter VSVG protein glycosylation. Whole cell HEK293T lysates transiently expressing VSVG-GFP (0.25ug) with Kir7.1 (0.25ug) or increasing concentrations of β 2AR (0.08, 0.125, 0.25ug) or MC4R (0.8, 1.25, 2.5ug), were treated +/- Endo H. As a control, VSVG-GFP only expressing cells were treated with a transport inhibitor (BFA-Monesin) and compared to untreated cell lysate. Anti HA Blots show receptor expression levels. (**) Endo H resistant, (***) Endo H sensitive. VSVG-GFP+3HA- β 2AR OE wells show that a 3-fold overexpression of β 2AR (0.75ug) can affect VSVG-GFP glycosylation. Data representative of three independent experiments.

β 2AR reduced Kir7.1 mediated whole cell currents

Given the specific effect of β 2AR on Kir7.1 glycosylation, we investigated whether this had an effect on the function of Kir7.1. To study the functional effect of the β 2AR mediated loss of Kir7.1 complex glycosylation, Kir7.1 whole-cell recordings were performed in the absence of any ligand in transfected HEK293T cells. Whole-cell patch clamp recordings were performed following Kir7.1 transfection, and over-expression of either β 2AR or MC4R. Since the Kir7.1 wild-type (WT) channel was found to have a small unitary conductance (Doring, Derst et al. 1998), we used a mutant form of the channel in which the methionine within the pore was replaced with an arginine typically found in the pore of other two-transmembrane domain Kir channels (Kir7.1-M125R). This mutant has been widely validated and used in multiple studies of Kir7.1 activity (Doring, Derst et al. 1998, Lewis, Bhawe et al. 2009, Raphemot, Lonergan et al. 2011). Kir7.1-M125R expressed alone compared to co-expression with MC4R yielded no differences in whole-cell current, which aligned with previous observations (Ghamari-Langroudi, Digby et al. 2015) (Fig. 3.3A-B). However, β 2AR caused a >50% reduction in Kir7.1 whole cell mediated currents (Fig. 3.3C-D). As Kir7.1-M125R channels exhibit barium sensitivity, activity of expressed Kir7.1 was taken as the total amount of current inhibited by barium at the end of the experiment. In general, reduced responses to changes in voltage were observed when Kir7.1 was co-expressed

with β 2AR (Fig 3.3D). As these experiments were performed in the absence of ligand, this suggested either glycosylation reduces the amount of functional channel at the surface of the cell, or directly alters channel function. We previously established that glycosylation is required for channel function (Chapter II), where the absence of glycosylation leads to a reduction in channel open probability. We wanted to further confirm that the reduced function observed was due to altered glycosylation and not β 2AR. We co-expressed β 2AR with our previously profiled glycosylation resistant mutant Kir7.1 M125R N95Q (Chapter II Fig. 3.3) to see if the receptor mediated any further reduction in channel activity. Current densities recorded at a test pulse of -180 mV were no different for Kir7.1-M125R-N95Q alone (110.8 ± 31.7 pA/pF) or Kir7.1-M125R-N95Q co-expressed with β 2AR (107.4 ± 21.8 pA/pF, $p = 0.8857$, unpaired t test) (Fig 3.4). These findings support our conclusion that β 2AR modulates Kir7.1 channel function by inhibiting complex glycosylation.

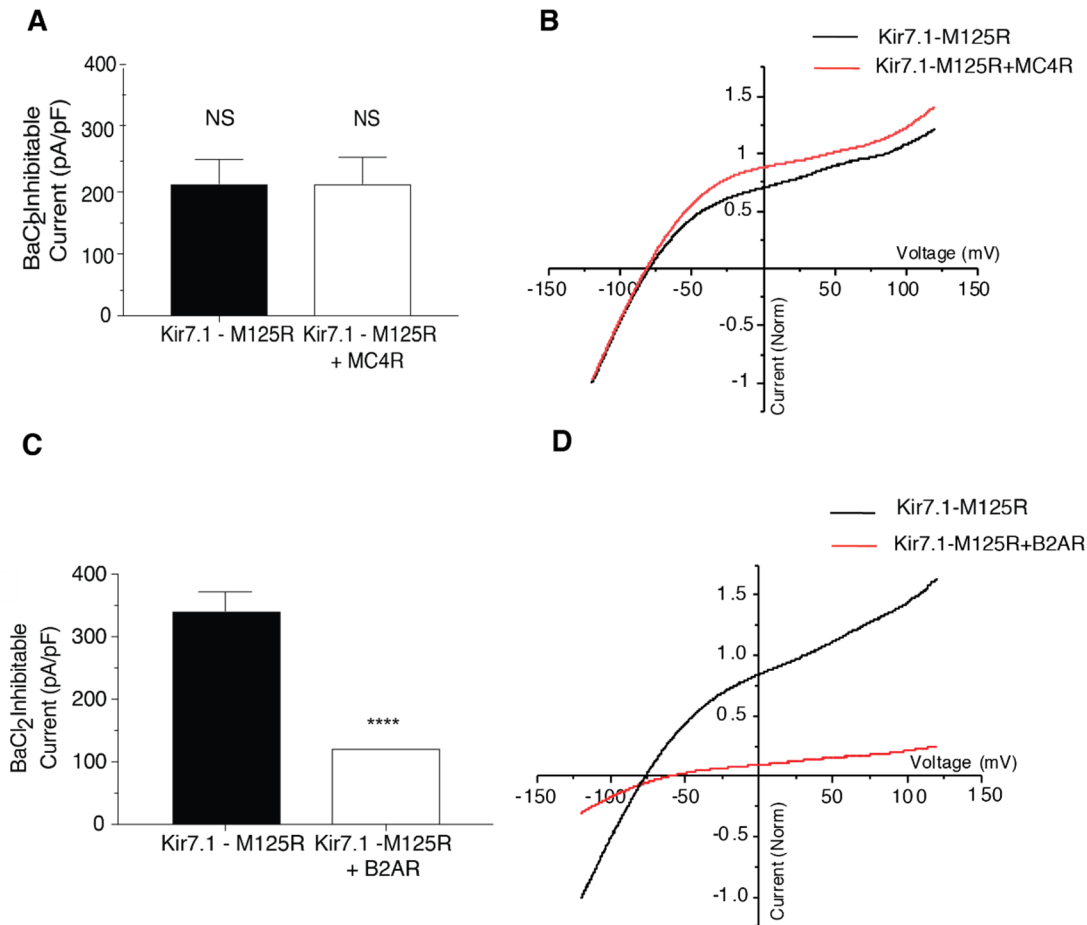


Figure 3.3 Coexpression of the β 2AR reduces total Kir7.1 mediated whole cell currents.

A. Whole cell patch clamp recordings from HEK293T cells transiently expressing Kir7.1-M125R only, versus Kir7.1-M125R co-expressed with MC4R (N=5). A representative current-voltage (I-V) relationship is shown in **B**. The Kir7.1-M125R mutant has a methionine in the pore of the channel mutated to an arginine typically found at the same site in most other Kir channels. This mutation significantly increases the amount of detectable currents from the channel, with increased sensitivity to barium. Therefore, the amount of current inhibited by barium at the end of the experiment, was recorded as the total current of Kir7.1-M125R from HEK293T cells. Mock transfected cells did not exhibit barium sensitive currents (data not shown). **C.** Kir7.1-M125R co-expressed with β 2AR showed a significant reduction in whole cell current in pA/pF, $p < 0.0001$ (N=7 cells), error bars represent S.E.M. **D.** Representative current-voltage (I-V) relationship of Kir7.1-M125R (●) in the presence or absence of β 2AR (●). Experiment performed by Daniel Swale

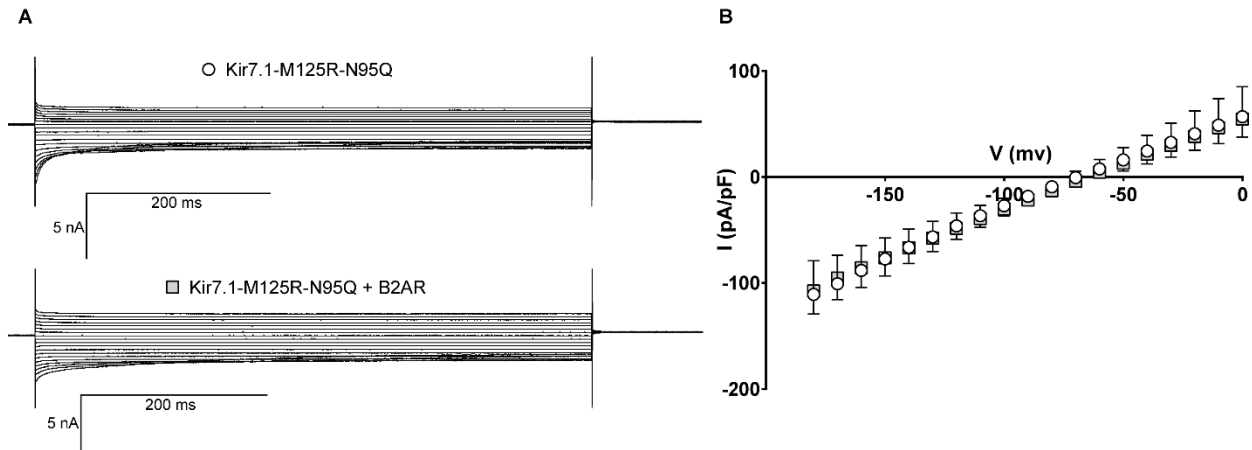


Figure 3.4 Co-expression with the β 2AR did not change Kir7.1-M125R-N95Q mediated whole cell currents.

A. Representative whole cell patch-clamp recordings from HEK293T cells transfected with Kir7.1-M125R-N95Q and Kir7.1-M125R-N95Q co-expressed with β 2AR. Cells were held at -60 mV and voltage steps were applied from -180 to 0 mV in 10 mV increments. **B.** The current density versus test potential (I - V) relationship for Kir7.1-M125R-N95Q and Kir7.1-M125R-N95Q co-expressed with β 2AR. Error bars on each symbol represent the mean \pm S.D ($N = 3$). Experiment performed by Ciria C. Hernandez

β 2AR mediated loss of complex glycosylation does not alter total Kir7.1 surface expression

We next investigated whether co-expression with β 2AR altered the amount of Kir7.1 trafficked to the cell surface. Cells co-expressing β 2AR or MC4R with Kir7.1 were biotin labeled, and assessed for cell surface expression by western blot analysis. This method allowed visualization of the different glycosylated forms of Kir7.1 reaching the cell surface. Surprisingly, Kir7.1 co-expression with β 2AR and loss of complex glycosylation did not cause a reduction in the percentage of Kir7.1 protein on the cell surface (Fig. 3.5B). MC4R likewise had no effect on Kir7.1 surface expression. Additionally, we assessed whether the ratio of complex to immature forms of Kir7.1 at the surface changed in the presence of these GPCRs (Fig. 3.5C). This remained unchanged, with β 2AR maintaining a <0.5 ratio of complex Kir7.1 glycosylation compared with the total glycosylated Kir7.1 expressed in the cell. A slight reduction in the ratio of surface

mature/immature Kir7.1 following MC4R expression was observed, but this trend was not significant over multiple experiments. These results therefore indicate that complex glycosylation is not required for surface expression of Kir7.1.

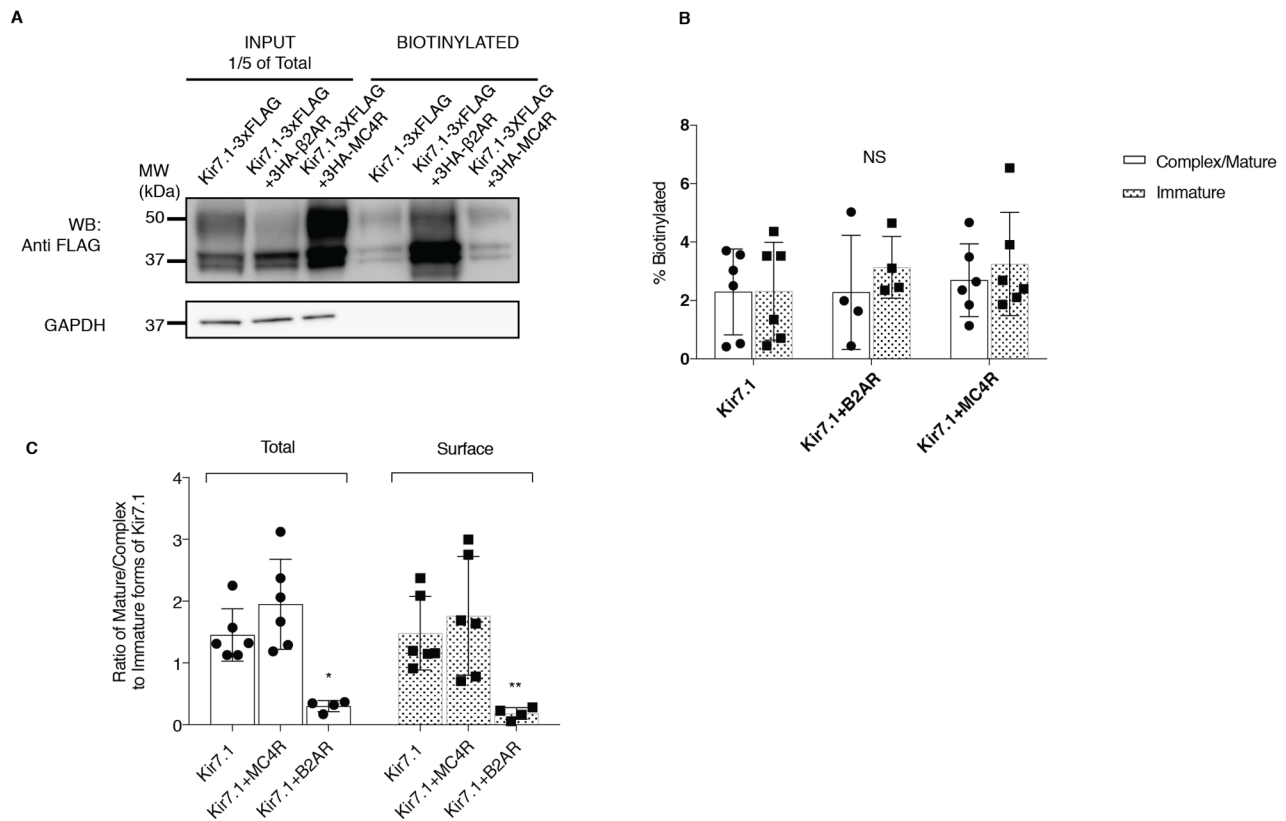


Figure 3.5 Co-expression of the β 2AR does not reduce the total amount of Kir7.1 at the plasma membrane, but alters the ratio of mature glycosylated forms of Kir7.1 at the surface. Transfection levels were as in Fig. 3.1. **A.** Western blot analysis of surface biotinylation of cells transiently transfected with 3xFLAG tagged Kir7.1 co-expressed with the 3HA- β 2AR or 3HA-MC4R. The experiment was performed as described in Chandrasekhar et al 2006(Chandrasekhar, Bas et al. 2006). Cells were labeled with Sulfo-NHS-SS-Biotin, and surface proteins were immunoprecipitated with streptavidin agarose beads. At least 45ug of pre-quantified total protein was immunoprecipitated and loaded per lane. 15ug (1/5) of total input protein was loaded for comparison. **B.** GAPDH control, indicated that cells remained intact throughout biotinylation. Surface quantification was determined by densitometry analysis of the mature/complex doublet, versus immature. Percent surface biotinylation was determined by dividing the values in the surface lanes by the input lane values multiplied by five. Percentages of complex or immature Kir7.1 at the surface were compared. See methods for data exclusion criteria. The error bars

represent S.D, with no significance observed between conditions determined by one-way ANOVA. C. Comparison of the ratio of mature to immature forms of Kir7.1 in whole cell lysates versus cell surface biotinylated proteins. Representative of 3-5 independent experiments, $p < 0.05$; $p < 0.005$, error bars represent S.D.

Other beta-adrenergic receptors also alter Kir7.1 glycosylation

We next sought to determine if suppression of complex glycosylation and function of Kir7.1 was unique to the $\beta 2AR$. For this, we first tested whether the $\beta 1$ and $\beta 3$ adrenergic receptors also mediated alterations in Kir7.1 glycosylation. We saw a similar reduction in the proportion of complex glycosylation when co-expressing these other beta-adrenergic receptors with Kir7.1 in HEK293T cells (Fig. 3.6A). Special care was taken to express the receptors at similar levels to allow adequate comparison for changes in glycosylation. $\beta 1AR$ and $\beta 3AR$ similarly reduced the ratio of Kir7.1 glycosylation as seen with $\beta 2AR$, with $\beta 1AR$ having a slightly lower value (Fig. 3.6B). This however may be due to receptor expression of $\beta 1AR$ being slightly higher than $\beta 2AR$ (Fig. 3.6A). These results indicate that our original observation was not specific to the $\beta 2AR$ receptor but shared by others GPCRs tested within the family. We also attempted to screen a larger panel of GPCRs for effects on glycosylation (MC1R, MC3R, OTXR), and did indeed see a reduction in Kir7.1 glycosylation ratio (Fig 3.7). While we were unable to attain consistent receptor expression levels for these receptors, the results suggest that many GPCRs suppress the complex glycosylation of Kir7.1

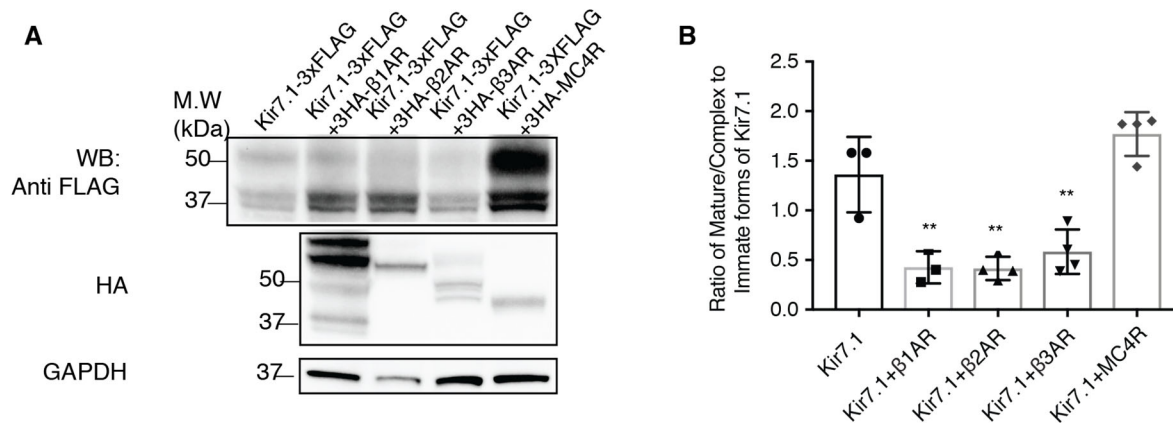


Figure 3.6 Multiple GPCRs inhibit Kir7.1 glycosylation. **A.** Western blot analysis of HEK293T whole cell lysates transiently transfected with 3xFLAG tagged Kir7.1, co-expressed with different 3xHA tagged beta-adrenergic receptors in the absence of ligands. Transfection levels were as in Fig. 3.1. Receptor expression was analyzed on a separate blot, with the loading control GAPDH shown. **B.** Densitometric analysis of the anti FLAG immunoblot of Kir7.1 shown in A, to quantify the ratio of mature to immature glycosylation $p < 0.005$. Data from 3 independent experiments were analyzed by one-way ANOVA in Graphpad Prism, with Tukey posttest for multiple comparisons with S.D.

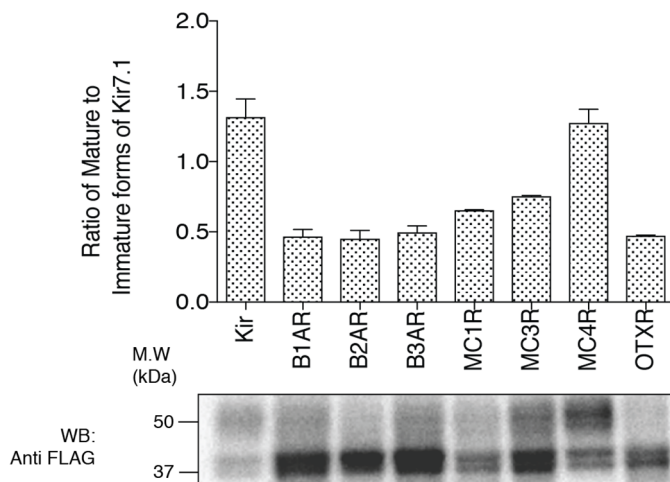


Figure 3.7 Other GPCRs alter Kir7.1 glycosylation to varying degrees. In a screen for the effect of other GPCRs on Kir7.1 complex glycosylation, cells were transiently transfected with Kir7.1-3xFLAG and various 3HA tagged GPCRs. 24 hours post transfection, samples were analyzed by western blot analysis. Receptor expression levels were not controlled in this experiment, and the ratio of Kir7.1 glycosylation was determined from two independent experiments. Bars indicate the mean ratio.

β2AR forms complexes with Kir7.1

With β2AR suppressing Kir7.1 complex glycosylation and not altering surface expression, we wanted to determine how the receptor might mediate its effects on Kir7.1. In chapter II, we established that Kir7.1 requires the Golgi export patch residues SY for complex glycosylation to occur. Thus, we wondered how these receptors may potentially block glycosylation from occurring in the Golgi. MC4R is the only GPCR tested that does not suppress Kir7.1 glycosylation, but has been shown to form complexes with the channel in HEK293T cells (Fig 3.8B) (Ghamari-Langroudi, Digby et al. 2015). Likewise, we co-expressed Kir7.1 with β2AR and co-immunoprecipitated (co-IP) complexes of the two proteins in HEK293T cells (Fig 3.8A). Interestingly, β2AR co-IP'd with only the immature form of the channels though small amounts of complex glycosylated forms were present in the cell. This suggests that the two proteins most likely need to be in contact for GPCR mediated suppression of complex glycosylation.

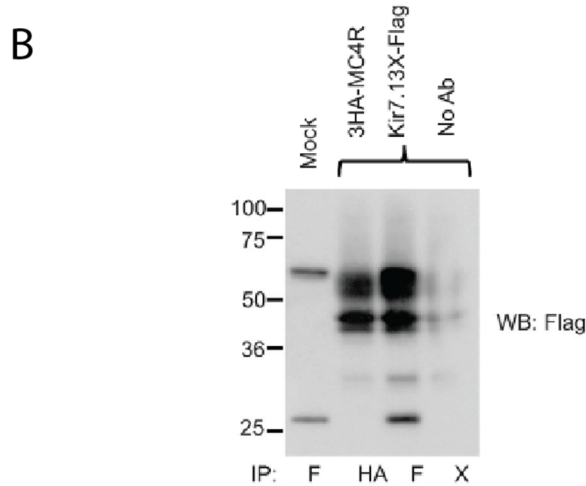
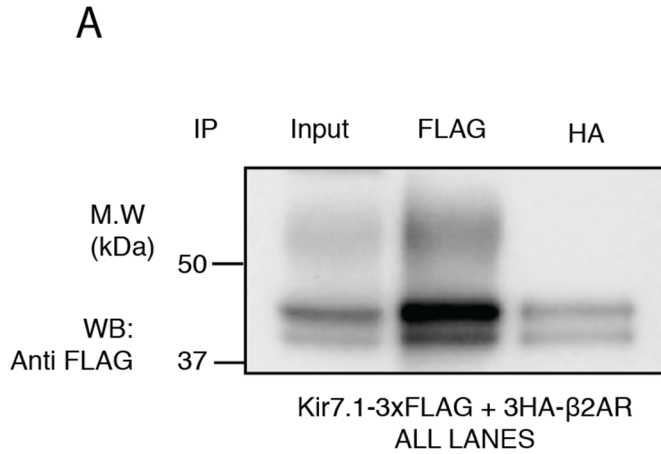


Figure 3.8 Kir7.1 co-immunoprecipitates with the β2AR.

A. Cells transiently transfected with Kir7.1-3xFLAG and 3HA-β2AR, were cross-linked with DSP prior to cell lysis. Anti-Flag M2 antibody or Anti HA antibody was used to immunoprecipitate the protein complex before analysis by western blot. Anti-Flag M2 antibody immunoprecipitated all forms of Kir7.1 expressed in the cell, which appears identical to the input lane. However, IP with the anti HA antibody to capture the β2AR, only co-immunoprecipitated the immature forms of Kir7.1. Data representative of three independent experiments. Detailed IP protocol outlined in methods. **B.** Image from (Ghamari-Langroudi, Digby et al. 2015). Cells transfected with Kir7.1-3xFLAG and 3HA-MC4R, were reversibly crosslinked with dithiobismaleimidoethane (DTME), and cells lysates were IP'd prior to western blot analysis. Blots shows that all forms of Kir7.1 co-IP with MC4R in HEK293T cells. F- Flag M2 antibody, HA- Anti HA antibody, X- no antibody control. Reproduced from Ghamari-Langroudi, Digby et al. 2015 with permission.

Discussion

G protein inwardly rectifying potassium channels (GIRKs) are Kir family members best understood to be regulated by GPCR signaling (Hibino, Inanobe et al. 2010, Luscher and Slesinger 2010). Our study reveals another mechanism by which GPCRs appear to regulate the function of another inward rectifier, Kir7.1, prior to cell surface expression. Our results demonstrate a highly specific effect of GPCRs on Kir7.1 glycosylation, which in turn directly regulates channel function. Specifically, we have shown that a reduction in the proportion of channel subunits undergoing complex glycosylation is mediated by expression of a wide variety of GPCRs. Further, we observed that the reduction in Kir7.1 whole cell currents when co-expressed with unliganded β 2AR (Fig. 3.3, Fig. 3.6) was indeed due to loss of complex glycans, an event occurring prior to trafficking of the channel to the cell surface. The MC4R has previously been reported to regulate Kir7.1, with agonists inhibiting and antagonists stimulating channel function, however expression of the MC4R did not reduce complex glycosylation of the channel, with or without the presence of ligand. Nonetheless, it is intriguing to note that the MC4R is one of the few GPCRs we examined that leaves channel glycosylation intact.

Of other non GIRK channels that interact with GPCRs, Kir4.1 is reported to interact with the calcium sensing receptor (CaR) GPCR within the kidney (Cha, Huang et al. 2011). Co-expression of CaR and Kir4.1 in HEK293 cells was found to reduce surface expression of the channel thereby limiting its activity. This was also reported to hold true for Kir4.2 and was found to be mediated by CaR Gq signaling and internalization by caveolin-1 (Huang, Sindic et al. 2007, Cha, Huang et al. 2011). This was not the case with β 2AR's effect on Kir7.1 wherein co-expression with β 2AR did not reduce Kir7.1 surface expression. In the case of CaR and Kir4.1, it was indeed

reported that mutant non-functional CaRs were unable to have an effect on Kir4.1 surface expression or activity (Huang, Sindic et al. 2007). We have not yet tested what function(s) of GPCRs are needed to alter glycosylation of Kir7.1.

While glycosylation is indeed not required for surface expression of ion channels such as Kir2.1 which is seemingly unglycosylated, we were surprised to see expression of immature forms of Kir7.1 at the cell surface. Particularly, the use of cell surface biotinylation allowed us to profile the different forms of the channel present at the surface and obtain a ratio of complex to immature plus unglycosylated forms. In co-expression with β 2AR, the ratio of complex glycosylation remained unchanged at the surface, indicating that GPCR expression, and inhibition of complex glycosylation, does not associate with altered trafficking of the channel. Furthermore, we have previously shown that these channels can exist as heterotetramers of unglycosylated, partially glycosylated, and fully glycosylated subunits (Chapter II Fig 2.5). Therefore, a change in the ratio of glycosylation most likely reflects a change in channel subunit composition. This would thereby contribute to subunit “poisoning” of Kir7.1 tetramers further influencing the reduction in channel activity observed (Fig 3.3C-D). We also observed complexes of β 2AR with the immature forms of Kir7.1 by co-immunoprecipitation (co-IP) in cells (Fig 3.8). After crosslinking, only the immature forms of Kir7.1 co-IP'd with β 2AR, though small amounts of complex Kir7.1 were present in the cell as observed in the input lane. This suggests that immature channels may be the predominant form in the cells when co-expressed with certain GPCRs.

Since β 2AR forms complexes with the immature form of the channel, this suggests a possible mechanism for the GPCR mediated loss of glycosylation. The protein titration experiment showed a progressive increase in immature forms of the protein, with increased expression of β 2AR. The amount of complex glycosylated Kir7.1 however was generally reduced. A possible

hypothesis is that β 2AR forms complexes with Kir7.1 early in the endoplasmic reticulum, and sterically blocks Kir complex glycosylation in the Golgi. Alternatively, the β 2AR could also differentially stabilize immature forms of the protein. The potential role of G proteins in the formation of these proposed receptor-channel complexes should be examined in future experiments.

The AP-1 gamma adaptin protein is the only protein reported in literature to bind to the Golgi export site in Kir channels. The role of this protein in glycosylation has not been investigated, or any other proteins that may bind to this site to mediate complex glycosylation. Our data suggest that GPCRs such as β 2AR are indeed preventing this crucial step of glycosylation from occurring, though the mechanism is unclear. It is plausible that GPCRs may indeed be blocking proteins from binding to this protein export site which we have shown is required for glycosylation.

Both the oxytocin receptor (York, Halbach et al. 2017), and the MC4R (Ghamari-Langroudi, Digby et al. 2015) have been shown to regulate Kir7.1 function at the membrane in a physiologically relevant manner, with the former mediated via a PIP2 intermediate. The mechanism(s) for regulation of Kir7.1 by MC4R remain to be determined, although the potential for Kir7.1 and MC4R to form complexes was shown by co-IP of tagged proteins from HEK293T cells (Ghamari-Langroudi, Digby et al. 2015). The inability of the MC4R to block complex glycosylation of Kir7.1, relative to other GPCRs we tested, is not understood, although it has been noted that the MC4R is one of the smallest GPCRs. One hypothesis is that MC4R may only form complexes with Kir7.1 post Golgi export, but this has not been tested. To study this, careful detection methods should be employed such as fractionation of membrane compartments followed by co-IP, or using super resolution microscopy combined with fluorescent tag protein

complementation.

The data shown here provide evidence for molecular regulation of Kir7.1 structure and function by GPCRs. Within tissues where Kir7.1 is highly expressed, large variations in glycosylation have been recently observed using a knock-in mouse expressing an HA tagged Kir7.1 channel (Cornejo, Villanueva et al. 2018). Notably, the study showed predominantly immature forms of the channel in the lung, a tissue with high levels of β -adrenergic receptor expression, whereas full complex glycosylation was observed in the trachea (Cornejo, Villanueva et al. 2018). No explanation for variation in glycosylation patterns between these two tissues was given, but our data may suggest a possible mechanism by which such changes could occur.

CHAPTER IV

Conclusions and Future Directions

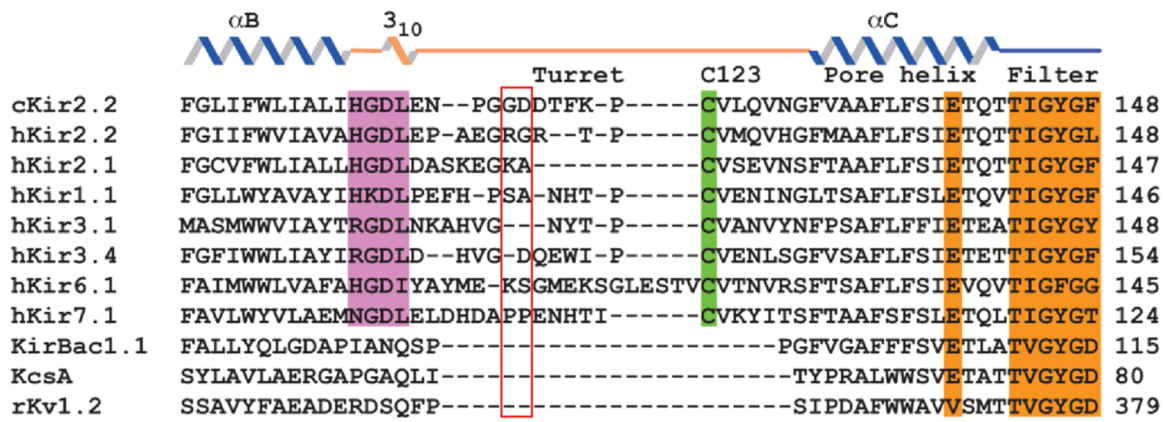
Probing the mechanism of glycosylation on channel function

In chapter II, we showed that glycosylation of Kir7.1 is essential for channel function. Specifically, the Kir7.1 N95Q mutant had a 40% reduction in channel amplitude versus wildtype, as well as a 50% reduction in open channel probability. These experiments were performed by single channel recordings and thus reflect the function of individual channels on the membrane of the cell. The glycosylation site N95Q was found by homology model analysis to be located in the extracellular loop within the turret region. In the Kir2.2 crystal structure the turret region is highly structured on the extracellular surface of the channel suggestive of a particular role in function (Fig. 4.1)(Tao, Avalos et al. 2009). The authors of that study postulate that turrets seem to “surround” and “protect” the pore gateway. Whether this is true has not been tested. The Kir7.1 homology model was built based on the Kir2.2 crystal structure. The turret region of Kir7.1 is not only two residues longer than Kir2.2, but it also contains two proline residues in succession which could potentially change the direction in which the loops point. The only Kir channel with a longer turret region than Kir7.1 is Kir6.1/6.2 (Fig. 4.1).

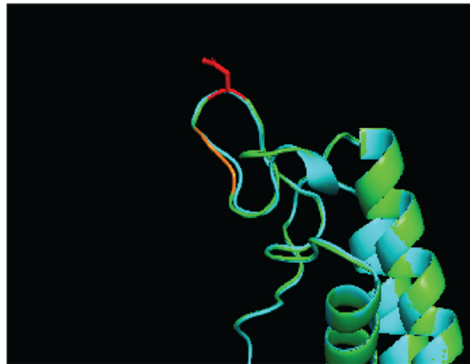
One method to probe the mechanism of glycosylation is by modeling the different conformations of Kir7.1 with the prolines as shown in figure 4.1. Next, site directed mutagenesis should be employed to determine whether changing the prolines to similar residues found in Kir2.2, would cause a loss of glycosylation or have some other impact on function. Additionally, whether these mutations affect channel assembly should also be explored. It is postulated that these

loops may either point towards the pore as shown in Kir 2.2, or the prolines induce a turn away from the pore (Fig. 4.1E). In the first scenario, the glycans which are large structures adding over 15Kd to the protein size as observed by western blot would tower over the pore. In the transient receptor potential 8 ion channel (TRPM8) which is activated by cold temperature, the presence of N-glycans in extracellular loops around the pore modulate the channel's responsiveness to temperature change (Pertusa, Madrid et al. 2012). Glycosylation may likewise have this effect on gating in Kir7.1. Alternatively, if the glycans point away from the pore, they will point to the abutting subunit in the tetramer, possibly linking the ion tetramer together to form a functional channel. Studying assembly of the Kir channel in non-denaturing conditions by western blot analysis would begin to answer this question, coupled with structure analysis of Kir7.1. This could be done by expressing either the WT or mutant channel in HEK293T cells, and analyzing whole cell lysates on a non-denaturing gel. If the absence of glycans reduces tetramer formation, a reduced number of high kDA running bands would be observed. This experiment may however be challenging to perform, due to membrane proteins forming aggregates that may not run cleanly through the gel in the absence of denaturing conditions. No published structures of Kir7.1 currently exist. However, preliminary evidence suggest that the N95Q mutant assembles properly as it co-immunoprecipitates with the WT channel, and with SY mutant subunits which do not have complex glycosylation (Chapter II Fig 2.5). The experiments outlined above would shed light on the exact mechanism.

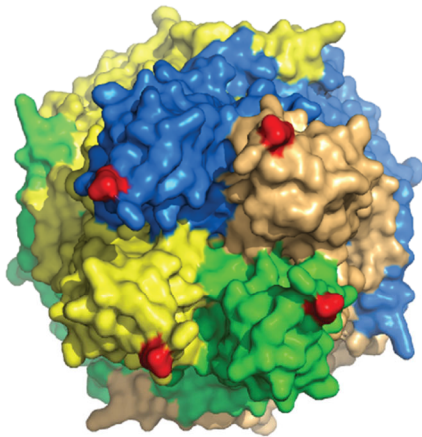
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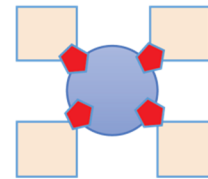
B



C



D



E

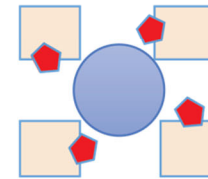


Figure 4.1 Glycosylation site of Kir7.1 is in the turret region and gates the pore.

A. Sequence comparison of turret regions within Kir channels. The proline residues present in Kir7.1 are shown in the red box. Image adapted from Tao et al 2009. **B.** Structural comparison of the turret region of Kir2.2 crystal shown in blue, and the homology model of Kir7.1 shown in green. The proline regions are highlighted in orange and the asparagine side chain glycosylation site is shown in red. **C.** Surface homology model of Kir7.1 looking downward into the pore. The red regions highlighted are the glycosylation sites gating the pore. **D** and **E**, representation of the potential turret loop conformations of Kir7.1. Loops may point towards the pore A, or away from the pore. Glycans are shown in red. Model of Kir7.1 created using SWISS-MODEL based on homology to Kir 2.2, and refined with PyMOL (see methods for structural models and protein alignments in Chapter V).

Retinopathy associated mutations in Kir7.1 that affect glycosylation

We discovered that a mutation in other parts of Kir7.1 outside of the glycosylation site can affect the ability of glycans to be added to the protein (See Fig. 2.6 in Chapter II). We found that the L241P Leber congenital amaurosis (LCA) associated mutation in Kir7.1 leads to a reduction in protein expression and a loss of complex glycosylation. We did not notice this pattern in other mutations tested, but our list was not exhaustive. Indeed, other mutations in Kir7.1 have been identified that are associated with retinopathies that should be assessed (see table 1). The location of the L241P mutation is in the C terminal region of the protein (Fig 4.2), and literature suggest that this may affect appropriate protein folding (Sergouniotis, Davidson et al. 2011). Whether the glycosylation site remains accessible in this protein is unknown.

Additionally, whether these mutations affect the ability of the protein to form stable tetramers may also provide a reason for loss of function. On a molecular level, most of these mutations have not been studied in detail. Given that this dissertation describes the ability of GPCRs to differentially alter the glycosylation of the channel, would any of these mutations affect this interaction? While the exact mechanism of Kir7.1-GPCR protein-protein interaction remains

unknown, these mutations may provide a clue into the structure function relationship of the channel.

Table 4.1 Sequence variants of Kir7.1 found within the human genome of individuals with diagnosed LCA

Nucleotide	Amino Acid	Prediction	Effect on Glycosylation	Reference
208G>T	Val70Phe	No effect	Not tested	(Sergouniotis, Davidson et al. 2011)
214G>T	Ala72Ser	Possible loss of function	Not tested	(Sergouniotis, Davidson et al. 2011)
344A>G	Glu115Gly	Possible loss of function	Not tested	(Sergouniotis, Davidson et al. 2011)
350A>G	Gln117Arg	Possible loss of function	No	(Sergouniotis, Davidson et al. 2011)
484C>T	Arg162Trp	Non-selective ion channel/Loss of function	No	(Hejtmancik, Jiao et al. 2008)
485G>A	Arg162Gln	Possible loss of function	No	(Sergouniotis, Davidson et al. 2011)
496C>T	Arg166X	Early stop codon		(Sergouniotis, Davidson et al. 2011)
722T>C	Leu241Pro	Possible loss of function	Yes	(Sergouniotis, Davidson et al. 2011)
827A>C	Glu276Ala	Possible loss of function	Not tested	(Sergouniotis, Davidson et al. 2011)
925G>T	Gly309Cys	Possible loss of function	Not tested	(Sergouniotis, Davidson et al. 2011)
295G>A	W53X	Truncated protein	NA	(Pattnaik, Shahi et al. 2015)

NA - Only first 52 amino acids expressed before stop codon.

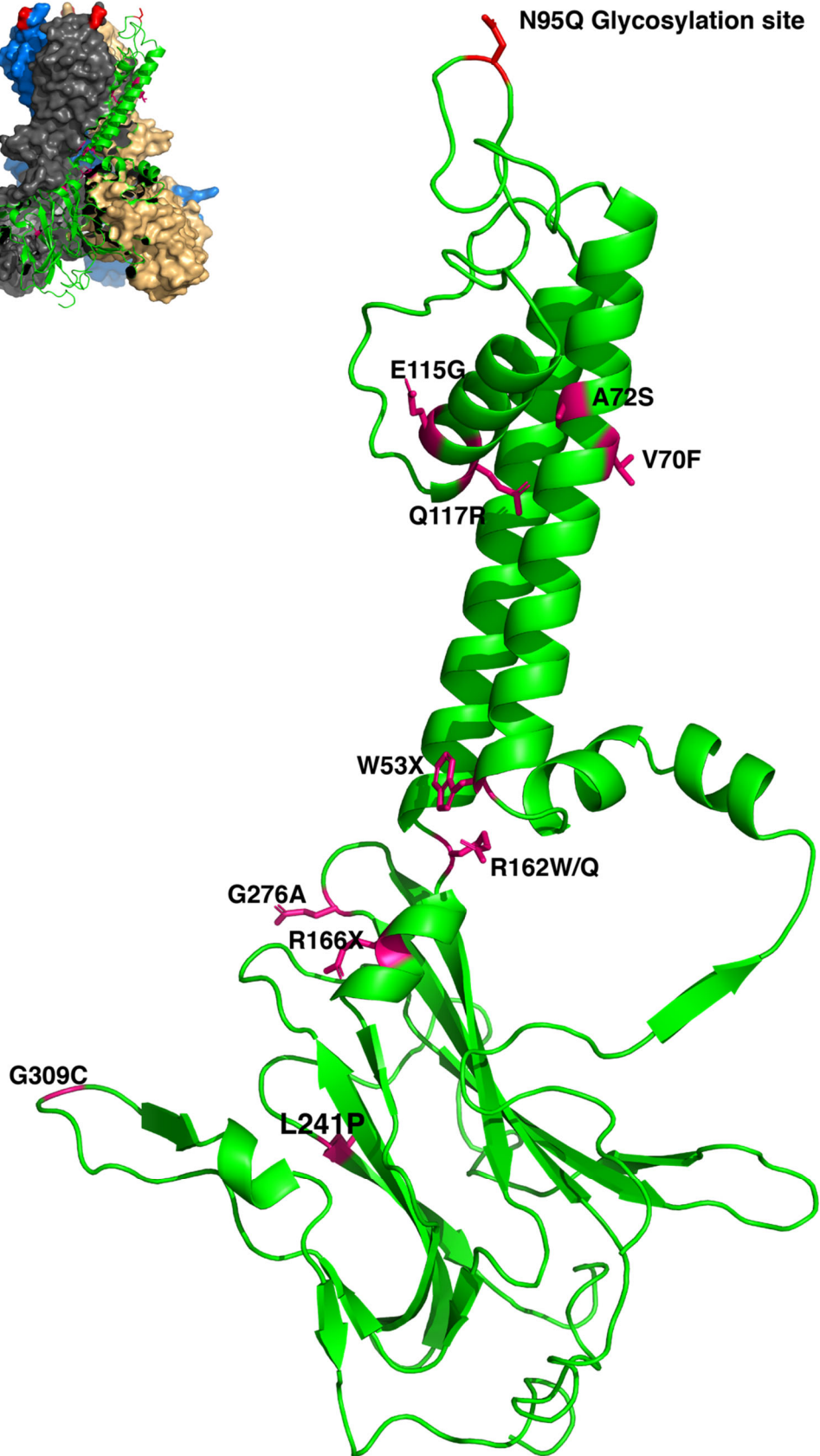
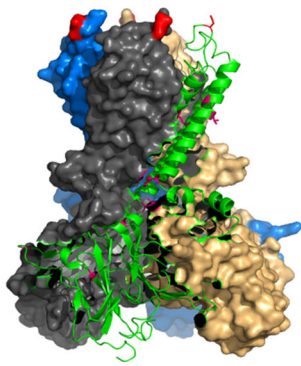


Figure 4.2 Homology model showing sequence variants of Kir7.1 found in the human genome. Individual subunit model of Kir7.1, with location of sequence variants shown in magenta with amino acid side chain. Amino acid number and variant change are labelled. Inset image shows a surface homology model of an entire Kir7.1 tetramer. The location of the individual subunit depicted with variants is shown within the tetramer. Model of Kir7.1 created using SWISS-MODEL based on homology to Kir 2.2 (see methods for structural models and protein alignments).

Determining the surface interface of GPCRs and Kir7.1

Kir7.1 interacts with and forms complexes with GPCRs as described in chapter III. The mechanism and sites of interaction remain unknown. Firstly, it must be determined whether other proteins exist in the complex with Kir7.1 and either B2AR and MC4R. This can be studied by isolating the complex after transfection and performing mass spectroscopy. For example, mass spectroscopy using “MudPIT” (Multi-dimensional Protein Identification Technology) analysis would be an initial method to catch all proteins that bind to Kir7.1 after crosslinking and immunoprecipitation. Next, individual proteins can be co-expressed with Kir7.1 to confirm whether an association exist with or without crosslinking to determine the strength of the interaction. However, given that GPCRs recruit proteins when activated, crosslinking may stabilize transiently interacting proteins. The presence of AKAPs or other scaffolding proteins should be ruled out before these detailed experiments are performed. AKAPs should readily be detected in the mass spectroscopy experiment.

Surface interface mapping is an emerging technique to allow the detection of direct molecular interactions between amino acids in proteins (Rappsilber 2011, Bullock, Sen et al. 2018). Briefly, this technique utilizes chemical crosslinkers that form irreversible linkages between amino acids. A cross linker can be selected to target either primary amines, cysteines or carboxylic acids. Once the intact proteins are crosslinked, they undergo trypsin digestion followed by liquid

chromatography and mass spectroscopy (LC-MS). Mass spectroscopy will detect the free uncrosslinked fragments in addition to the crosslinked peptides. The peptides are then identified against a protein database to detail a list of linked and or free fragments. Using either a crystal structure or homology model of both proteins, the interacting peptides can then be identified. To confirm the surface interface regions, mutational analysis would then be performed to determine if changing specific amino acids or domains on the protein would affect protein complex formation. These efforts are currently underway by a collaborator, Dr. Glenn Millhauser, to determine the surface interface of Kir7.1 and MC4R. These experiments and others would be greatly improved if structures for Kir7.1 and MC4R are resolved. As the B2AR receptor was one of the first GPCRs crystalized, it could serve as a starting point to probe the Kir7.1/GPCR protein-protein interaction (Rasmussen, Choi et al. 2007).

While no current structures of Kir7.1 have been published, structures of other family Kir's are available- KirBac 1.1, Kir2.2, Kir3.1, Kir6.2 (Nishida, Cadene et al. 2007, Clarke, Caputo et al. 2010, Lee, Ren et al. 2016, Li, Wu et al. 2017). None of these sequences are currently of human Kir's, but are of highly conserved sequences from other genomes. Membrane proteins are notoriously difficult to obtain crystals for structural determination, but other methods such as Cryo-EM have provided simpler alternatives for this work. Dr. Alys Peisley in the Cone laboratory has purified Kir7.1 from insect cells, and is currently determining its structure using Cryo-EM. Ultimately, the lab hopes to move on to determine a Kir7.1-GPCR structure.

Possible mechanisms of GPCR mediated inhibition of Kir7.1 complex glycosylation

While MC4R did not alter the complex glycosylation of Kir7.1, all other GPCRs tested affected channel complex glycosylation. The mechanism by which this inhibition occurs currently remains unclear. Proteins typically traffic through the secretory pathway from the endoplasmic reticulum to the Golgi complex before vesicle packaging and relocation to various subcellular compartments. Membrane proteins are exported and inserted into the membrane. Immature glycosylation occurs in the ER, and complex glycans are added in the Golgi. In the case of GPCRs blocking complex glycosylation of Kir7.1, addition of complex glycans is either being blocked at a specific point in the Golgi, or could result from the proteins undergoing a Golgi bypass mechanism.

Some proteins have been shown to traffic to the membrane via an unconventional route known as Golgi-bypass. In this case, proteins are packaged in vesicles at the ER, and are routed directly to the membrane. This pathway is typically induced in episodes of cellular stress in cells where it has been studied (Piao, Kim et al. 2017, Kim, Gee et al. 2018). For example, in epithelial Madin-Darbt canine kidney (MDCK) cells, proteins avoided the Golgi and trafficked to the membrane in the presence of BFA (Tveit, Akslen et al. 2009). BFA inhibits transport of proteins to Golgi, and at high concentrations will result in Golgi complex collapse. Addition of an ER retrieval signal peptide to trap cargo in the ER also resulted in Golgi bypass and expression at the membrane in untreated cells. In fact, ER stress mechanisms such as the unfolded protein response have been also been shown to be involved in this pathway. We tested whether expression of Kir7.1 and GPCRs induced ER stress by looking for the expression of ER stress markers XBP1s and cell death marker Chop (Fig. 4.3). ER stress marker expression levels were not greater when GPCRs were expressed with Kir7.1 versus Kir7.1 expressed alone. Additionally, VSVG-GFP, a widely

studied glycoprotein was still fully glycosylated when co-expressed in states where GPCRs affected Kir7.1 glycosylation (Chapter II, Fig 2.1). This indicated that the Golgi bypass pathway was most likely not being induced, and that another mechanism was responsible for the loss in glycosylation.

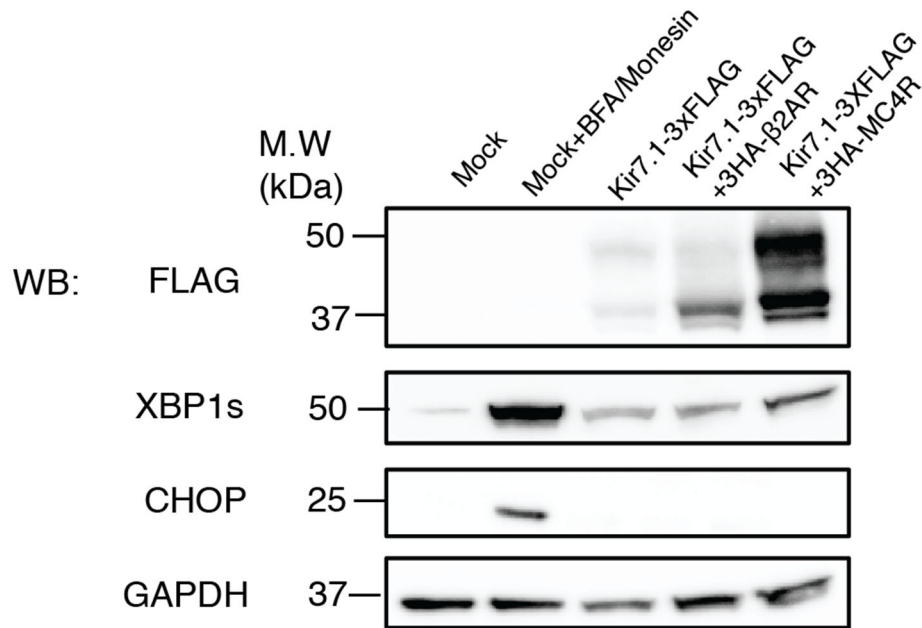


Figure 4.3 Co-expression of Kir7.1 and GPCRs does not lead to ER stress in induction above Kir7.1 expressed alone

Western blot analysis of cells expressing Kir7.1 alone or co-expressed with B2AR or MC4R for the expression of ER stress markers. Mock transfected cells were treated with the transport inhibitor BFA/Monesin to induce ER stress, and western blot analysis was performed for the ER stress markers XBP1s (Xbox binding protein 1 spliced isoform) or CHOP (C/EBP-homologous protein). Data representative of three independent experiments.

Complex glycans are added as proteins traffic through different regions of the Golgi complex. Membrane proteins are classically thought to traffic progressively through each Golgi compartment before transport to the cell surface (cis-medial-trans Golgi network). The point at which major complex glycans are added is unclear for ion channels. Endo H resistance is conferred with the addition of an N-acetylglucosamine sugar to the mannose-oligosaccharide added in the ER (Grieve and Rabouille 2011). The point at which this occurs for different proteins also remains unknown. A recent study of the Golgi export site in the Kir2.1 channel was suggestive that this addition occurs between the cis and trans Golgi compartments. As described in Chapter II, Kir channels share a Golgi export site in which the serine and tyrosine (SY) residues in some channels are key for surface expression (Ma, Taneja et al. 2011, Li, Ortega et al. 2016). Deletion of these residues trap the channels in the cis and trans Golgi compartments. Researchers found that placement of an artificial N-linked glycosylation site in the Kir2.1 Golgi export mutant resulted in a protein with core glycans, but lacked complex glycosylation. This suggest complex Endo H resistant glycans are added later in these compartments before export to the membrane. Kir7.1 shares Golgi export patch residues (Chapter II, Fig 2.4), the SY mutant similarly lacks complex glycosylation. Our preliminary data suggest that this mutant is not trapped in the Golgi but is indeed trafficked to the surface (not shown). The key question to be answered is whether GPCRs interact with Kir7.1 in the ER or at this point in the Golgi, and block the access of enzymes to allow complex glycosylation.

This can be tested through multiple experiments, but currently there are alternate hypotheses to how proteins transit through and are exported from the Golgi. In addition to progressive trafficking through the Golgi network, another plausible hypothesis is the “rapid partitioning in a mixed Golgi” model (Patterson, Hirschberg et al. 2008, Lippincott-Schwartz and

Phair 2010, Glick and Luini 2011). Simply, as shown in Figure 4.4 this hypothesis posits that proteins trafficking to the Golgi can partition into one of the Golgi stacks (cis, medial or trans) and instead of forward progression through the entire Golgi complex, exit and traffic to the membrane from the stack of partitioning, i.e. proteins can be exported directly to the membrane from cis, medial or trans stacks . This hypothesis was tested by Patterson et 2008, when they studied the kinetics of VSVG-GFP exit from the Golgi in pulse chase experiments. Classical Golgi transport suggest a lag in progression time as proteins move through the entire stack before exit. However, after a 5 minute pulse labeled proteins were observed leaving the Golgi exponentially with no lag observed. Additionally, they observed VSVG-GFP proteins scattered throughout the Golgi by gold particle labelling, with no enrichment in the most trans lying stack that would classically serve as the export stack. The authors ultimately put forward the hypothesis that VSVG can exit at any point in the Golgi complex.

As it relates to Kir7.1 and GPCRs, it is possible that GPCRs may prevent complex glycosylation by promoting an early Golgi exit before reaching later stacks where complex glycosylation occurs. This can be tested using pulse chase experiments and super resolution microscopy of trafficking in the Golgi. Using fluorescently tagged proteins, complexes of Kir7.1/ β 2AR or Kir7.1/MC4R could be tracked actively leaving different Golgi compartments. Additionally, membrane organelles can be isolated by differential centrifugation to determine where Kir7.1 and GPCRs exist as a complex, as well as changes in Kir7.1 glycosylation states as it traffics through the secretory pathway. While there are reports that MC4R is complex glycosylated, this was not observed in our experiments, nor does β 2AR itself have complex glycosylation (Figure 4.5). Indeed, the alternate Golgi hypothesis may be plausible as these GPCRs do not compete for glycosylation enzymes.

While we did not observe complex receptor glycosylation, the presence of glycans on β 2AR is reported to play a role in receptor dimerization (Li, Zhou et al. 2017). β 2AR has three N-glycosylation sites, with N6, and N15 identified as the key residues playing a role in homodimerization of the receptor. In fact, mutation of two residues at the dimer surface interface on β 2AR mimicked the glycosylation mutant reduction in dimerization. The interface between Kir7.1 and β 2AR or MC4R has not been identified, but a role for receptor glycosylation in the interaction remains unexplored. This could be tested by determining whether glycosylation mutants of β 2AR are still able to inhibit the complex glycosylation of Kir7.1. Additionally, the dimer surface interface residues of β 2AR were found to form a salt bridge between the two receptors (Li, Zhou et al. 2017). If a β 2AR protein with these mutations is unable to alter channel glycosylation, this would indeed indicate a possible receptor interface that facilitates this interaction.

The stoichiometry of receptor dimerization and complex formation with Kir7.1 also remains unexplored. It is unknown whether receptors assemble 1:1 with Kir subunits i.e, one Kir tetramer would have 4 receptors bound, or whether a receptor dimer assembles with one Kir tetramer. Cryo-EM experiments would provide visual proof of Kir/receptor complex assembly in the membrane.

A

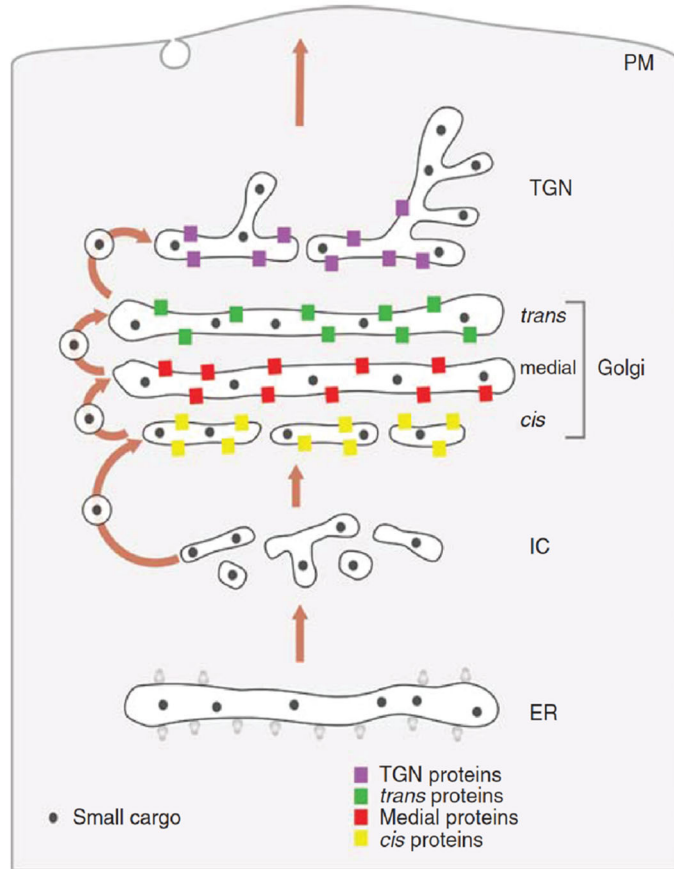
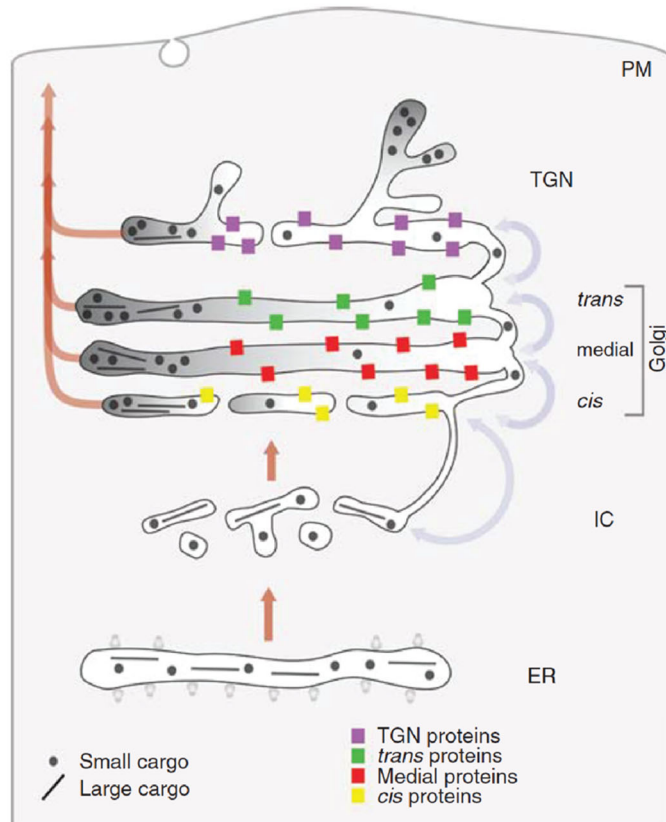


Figure 4.4 Models of Golgi Trafficking

A. Progressive/directional protein trafficking model of Golgi transport. Proteins move progressively through each partition, and exit through the trans-Golgi network to the membrane.

B. Rapid partitioning in a mixed Golgi model. Proteins can partition into any Golgi compartment, and exit directly to the plasma membrane, without transitioning to the next compartment in sequence. Images adapted from Glick & Luini 2011, Cold Spring Harb Perspect Biol. Reproduced with permission.

B



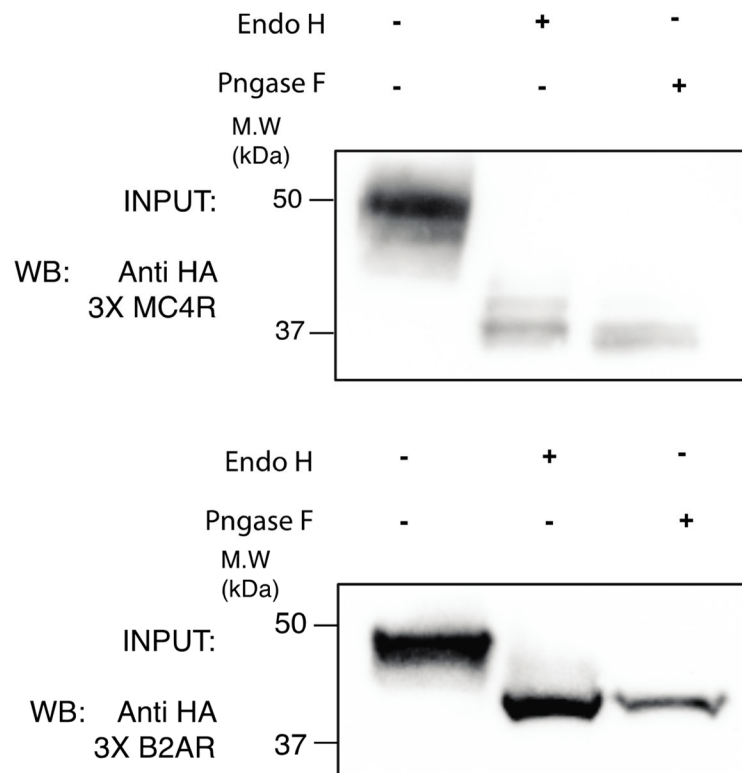


Figure 4.5 MC4R and B2AR do not undergo complex glycosylation

Western blot analysis of cells expressing MC4R or B2AR and treated with either Endo H and Pngase F. Whole cell lysates of HEK293T cells expressing either receptor were treated with Endo H to determine core glycosylated proteins, or Pngase F to cleave all glycans. Proteins were analyzed by western blot analysis.

The Golgi export site of Kir channels is also reported to be the binding site of the AP-1 adaptor protein. AP-1 adaptor protein binding is required for export of Kir2.3, and Kir4.1/5.1 channels to the cell surface (Li, Ortega et al. 2016). The authors in this study also suggest that Kir7.1 does not share a strong export patch with the other channels and thus may not be needed for trafficking, which our preliminary data supports. Given that the SY residues are essential for Kir7.1 glycosylation, the AP-1 adaptor proteins or other proteins which bind the site may be

essential to facilitate channel glycosylation. Mass spectroscopy analysis experiments of Kir7.1, proposed in an earlier section, could reveal adaptor type proteins which bind this site. Whether GPCRs block access to these proteins in order to prevent trafficking through the appropriate glycosylation pathway could then be tested.

Role for inhibition of Kir7.1 glycosylation in physiology

The GPCR mediated inhibition of Kir7.1 glycosylation was discovered while probing the original Kir7.1/MC4R interaction. In hypothalamic neurons, Kir7.1 functions to aid MC4R's role in modulating food intake and energy homeostasis (Anderson, Ghamari-Langroudi et al. 2018). These data show that activation of MC4R leads to closure of Kir7.1 and inhibition of MC4R by an inverse agonist promotes channel opening. These experiments were performed using an electrophysiological slice preparation. Erica Anderson a graduate student in the lab took this a step further by deleting Kir7.1 within MC4R containing hypothalamic neurons. Within the slice preparation, the MC4R agonist failed to cause the neuronal depolarization that was observed when Kir7.1 was expressed. Furthermore, these animals had increased weight gain compared to controls indicating a role for Kir7.1 in MC4R's modulation of energy homeostasis. Kir7.1's expression is quite low within the paraventricular nucleus (PVN) of the hypothalamus where MC4R is expressed, and this indicates the sensitivity of the system to the presence of the channel.

Within the uterine smooth muscle, Kir7.1 activity regulates excitability. Specifically, Kir7.1 expression increases in mid-gestation promoting uterine quiescence, and expression levels fall significantly prior to labor promoting parturition (McCloskey, Rada et al. 2014). Expressed channels also appear to be maintained in an active state, with channel expression inversely proportional to uterine excitability. These studies of Kir7.1 in the brain and the uterus represent

two different mechanisms by which Kir7.1 activity can be regulated. This research identifies a third mechanism, that GPCRs can limit post translational glycosylation of Kir7.1 thereby resulting in a channel with reduced activity on the surface of the cell.

As described in chapter I, GPCRs that couple through Gq can regulate Kir activity by depletion of PIP₂. Kir7.1 was recently reported to interact with the oxytocin receptor (OXTR) in retinal pigment epithelial cells (York, Halbach et al. 2017). Activation of OXTR led to a reduction in Kir7.1 recorded currents from transfected HEK293 cells and also in RPE cells. This was also shown to be mediated by PIP₂ as expected. In the uterus, oxytocin through OXTR promotes uterine contraction, which would also occur around the time of diminished Kir7.1 expression. Indeed, the possibility exists that OXT could also function to diminish activity of any remaining Kir7.1 channels in the smooth muscle. Additionally, given our findings, expression of OXTR could also lead to a reduction in the complex glycosylated Kir7.1 channels at the surface. Thus, Kir7.1 could be regulated by multiple mechanism based on the signaling within the cell. A proposed model of Kir7.1 regulation is shown in figure 4.6. Changes in Kir7.1 complex glycosylation could be observed in native tissues supporting this hypothesis. Cornejo et al created an HA-tag knock-in mouse for Kir7.1 and observed large variations in glycosylation between tissues, such as trachea and lung tissue. Complex glycosylation was observed in the trachea but significantly diminished in the lung (Cornejo, Villanueva et al. 2018). B2AR expression is quite high in the alveolar epithelium of lung in the smooth muscle lining the airway where it promotes ion transport via PKA activation (Mutlu and Factor 2008). Loss of Kir7.1 is not only lethal at birth, but has also been linked to retardation of trachea and lung development in mice (Villanueva, Burgos et al. 2015). While a role for Kir7.1 in the lung is not clear, it is indeed likely that a GPCR may be responsible for the reduction in glycosylation observed. Using this mouse model, complexes of Kir7.1 could

be isolated from different tissues to test this hypothesis.

In conclusion, regulation of complex glycosylation of Kir7.1 by GPCRs is a new and novel mechanism that needs to be further studied. We determined that glycosylation of Kir7.1 is essential for function, with Kir1.1 being the only other GPCR for which a role for glycosylation in function has been confirmed. Additionally, we have found that MC4R currently remains the only GPCR that does not induce changes in Kir7.1 glycosylation, whereas other GPCRs tested do. However, MC4R is the only GPCR reported to both interact and regulate Kir7.1. This receptor's interaction with Kir7.1 thus appears unique and needs to be explored further. Our research shows that multiple mechanisms can regulate the function of Kir7.1, and indeed identifies a new mechanism of interaction between GPCRs and ion channels.

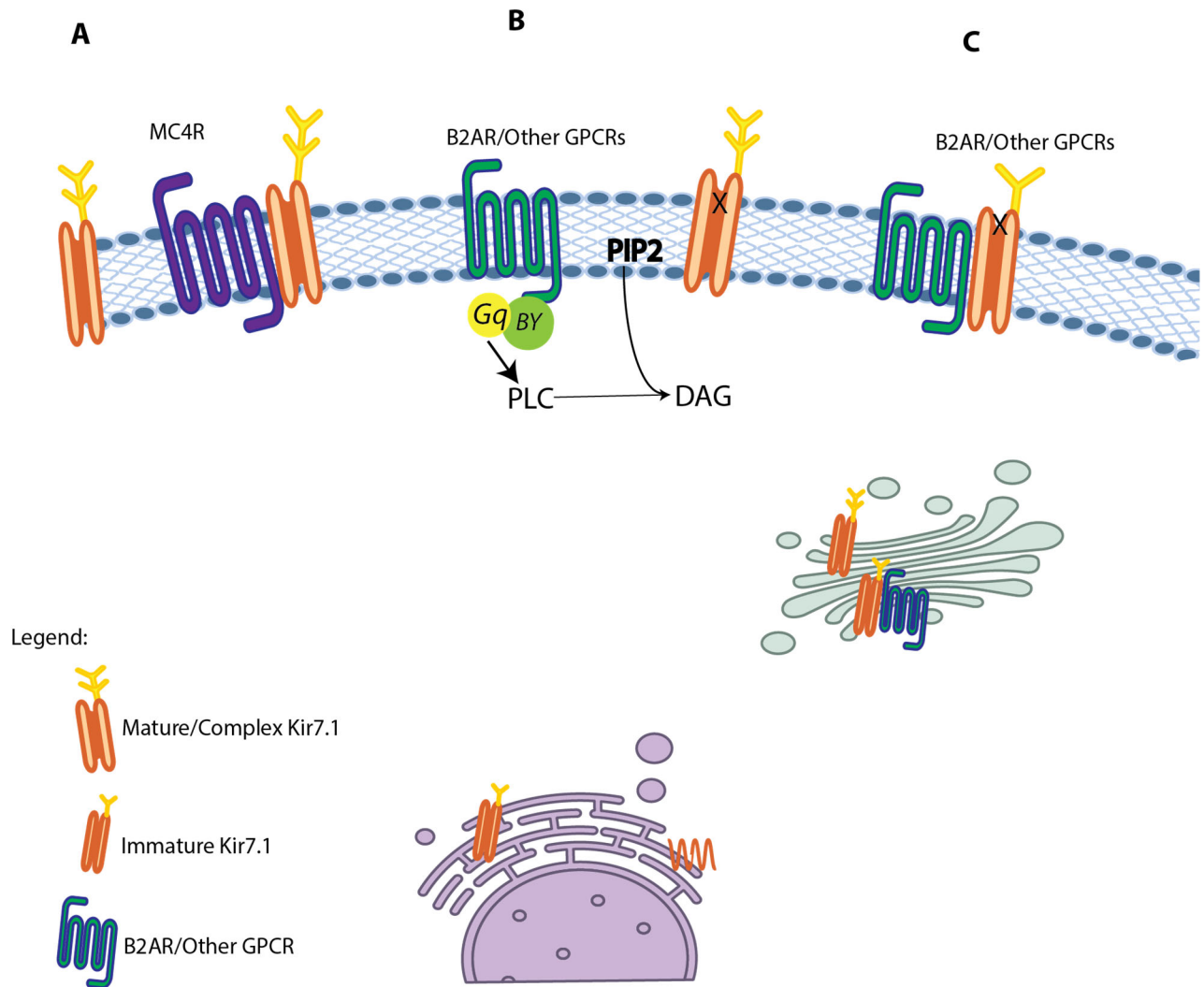


Figure 4.6 Mechanisms of Kir7.1 Regulation

- A.** MC4R couples to Kir7.1 to induce channel closure when MC4R is activated by α MSH. The inverse agonist AGRP promotes channel opening.
- B.** Gq coupled receptors such as OXTR activate PLC which converts PIP₂ to DAG. PIP₂ depletion leads to channel rundown.
- C.** Other GPCRs interact with Kir7.1 possibly in the Golgi, and prevent complex glycosylation. Ion channels reaching the surfaced have reduced open channel probability.

CHAPTER V

METHODS

Cell lines and cell culture

HEK293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), with high glucose, L-glutamine and phenol red; supplemented with 10% fetal bovine serum (Atlanta Biologicals), and 1% antibiotic-antimycotic (Thermo Fisher Scientific). Cultures were maintained in 5% CO₂ environment at 37°C. Cos1 cells were similarly cultured to HEK293T cell lines with medium containing sodium pyruvate. The protein transport inhibitor cocktail from BD Biosciences was used at 0.5X and cells were incubated for 9 hours prior to harvesting.

Transfection

Plasmid DNA constructs were transfected into HEK293T or Cos1 cells at 70-80% confluency using LipD293 reagent (SignaGen) according to manufacturer's instructions. Cells were allowed to grow for 24 hours before harvesting. If cells were kept for 48 hours, media was changed after 24 hours.

Cell surface biotinylation

Cell surface biotinylation was performed as described in Chandrasekhar et al 2006(Chandrasekhar, Bas et al. 2006). Briefly, HEK293T cells were transiently co-transfected at 70-80% confluency with Kir7.1-3XFLAG and either 3HA-β2AR or 3HA-MC4R. 24 hours post transfection, cells were washed three times with phosphate buffered saline with calcium and

magnesium (PBS²⁺). Cells were then incubated with 1mg/mL of Biotin-SS-sulfo (ApexBio) in PBS²⁺ twice for 15 minutes each. Excess biotin was quenched with two short washes followed by two 15-minute incubations with 100mM glycine in PBS²⁺. Cells were washed with PBS, then lysed with modified RIPA buffer (see western blot protocol). The protein samples were quantified, and 25uL of PierceTM high-capacity streptavidin agarose beads (Thermo Scientific) were incubated overnight with 75ug of protein lysate. The supernatant was removed and the beads were washed three times with lysis buffer. The beads were first eluted with 2x LDS with 200mM DTT, then with 100mM DTT in lysis buffer. Both elutions were combined, and loaded on a 10% BoltTM Bis-Tris Plus gel (Invitrogen) next to 1/5 of the input (15ug of total cell lysate). Western blot analysis was continued as described. Samples were quantified as described in the next section (Western blot and quantitative analysis). Input lane values post densitometry were multiplied by five before comparison to biotinylated lane values. Biotinylated values were taken as a percentage of the input lane values to derive the amount of protein at the cell surface. Samples with % biotinylated values of less than 1 were excluded from analysis. A minimum of four experiments were used for analysis.

Western blots and quantitative analysis

Post transfection, cells were lysed in a modified RIPA buffer containing 20mM Tris-HCL pH8.0, 150mM NaCl, 1% Triton X-100, 2mM EDTA and protease inhibitor cocktail (Sigma/Roche). Cells were scraped and incubated on ice with rocking for 30 minutes followed by a 10,000 rpm centrifugation for 10 minutes. The supernatant was collected and protein quantified using a Pierce BCA Protein Assay Kit. Lysates were prepared for denaturing gel electrophoresis by mixing with NuPAGE[®] LDS Sample Buffer (4X) (Invitrogen) with 400mM DTT to a final

concentration of 1X LDS, 100mM DTT. Invitrogen Bolt™ 10% Bis-Tris Plus precast polyacrylamide gels were used to run 10-15 ug of protein at 200V for 40 minutes using Bolt™ MOPs running buffer. Gels were run with Bolt™ running buffer with Bolt™ Antioxidant added to the first chamber of the mini gel tank (Thermo Fisher Scientific). A Biorad protein ladder, Kaleidoscope, Dual Color or All Blue was also included on each gel. The gels were then transferred to PVDF membrane (Millipore) using Bolt™ Transfer buffer, with the Trans-Blot Turbo™ Transfer system from Biorad. Membranes were blotted with 5% non-fat dry milk in phosphate buffered saline with 1% Tween-20 for 30 minutes, prior to overnight incubation with antibodies. Blots were washed a minimum of three times for 10 minutes each before imaging with SuperSignal™ West Dura Extended Detection Substrate using the Biorad ChemiDoc™ Touch Imaging System. Images with samples of interest within the dynamic range were chosen for further quantitative analysis using the Biorad Image Lab™ Software. Ratios of mature to immature glycosylation were obtained by dividing the intensity of the upper 50kD doublet, by the lower 35kD doublet intensity. No normalization was performed on these values as the ratios are independent of protein loading. For comparison of receptor expression levels, intensities recorded were normalized to the loading control GAPDH. Data was analyzed using GraphPad Prism software. A one or two-way ANOVA statistical test was used to determine significance ($p < 0.05$), with Tukey post-test for multiple comparisons.

Glycosidase treatments

Membrane proteins traffic through the secretory pathway where post translational modifications such as glycans are added before reaching the cell membrane. Channel glycosylation occurs initially in the endoplasmic reticulum (ER), before more complex glycans are added in the Golgi.

Glycans added in the ER versus the Golgi can be differentiated using endoglycosidases. Endoglycosidase H (Endo H) cleaves most immature N-linked glycans which are typically added in the ER. Once proteins traffic through the Golgi where more complex glycans are added, the protein becomes resistant to Endo H. The enzyme PNGase F however, is able to remove all N-linked glycans present on the protein, both immature and complex glycosylation. We investigated protein glycosylation using the endoglycosidases Endo H and PNGase F from New England Biolabs (NEB). Briefly, cells transiently transfected with Kir7.1-3XFLAG or Kir7.1 Δ SY-3XFLAG were harvested and protein was quantified as previously described. 50ug of lysate was denatured by addition of 10x glycoprotein denaturing buffer (NEB) followed by heating for 10 minutes at 55°C. Samples being treated with Endo H were mixed with Glycobuffer 3, and 2 ul of Endo H enzyme. The mixture was incubated for 1.5 hours at 37°C in a thermocycler. Samples treated with PNGase F were mixed with GlycoBuffer 2, plus 1 uL of the enzyme and also incubated for 1.5 hours at 37°C. The treated samples were then mixed appropriately with 4X NuPAGE LDS buffer with 400mM DTT, and analyzed by western blot analysis as described. Samples were compared to untreated lysates and observed for band shifts in migration.

Expression constructs

Plasmids with full length cDNA for the GPCRs β 1AR, β 2AR, β 3AR and MC4R were obtained from the cDNA Resource Center (cdna.org), with 3xhemagglutinin (3xHA) N-terminal tags in the pcDNA3.1+ expression vector. The full length cDNA sequence for KCNJ13 (AJ006128.1) was cloned into the pCiNEO expression vector (Promega) with a C terminal 3XFLAG tag (Sigma). To create the HA-Kir7.1 construct, the full length cDNA sequence for KCNJ13 was

cloned into a pcDNA3.1+ vector with a single N-terminal HA tag. To create mutations in Kir7.1, primers were designed using the NEBaseChanger online tool, and ordered from Sigma. Mutagenesis was performed using NEB Q5 Site-Directed Mutagenesis kit (New England Biolabs). For electrophysiology, the cDNA sequence of Kir7.1 was cloned into pcDNA5/TO expression vector (Thermo Fisher Scientific) without tags. The VSVG-GFP construct was provided by the Kenworthy lab at Vanderbilt University.

Antibodies

Western blotting was performed using the following antibodies: Monoclonal ANTI-FLAG[®] M2-Peroxidase (HRP) antibody produced in mouse (Sigma-Aldrich)1:10,000; HA-tag (6E2) Mouse mAb (HRP conjugate) (Cell Signaling Technology)1:5000; GAPDH (D16H11)XP[®] Rabbit (HRP conjugate)1:5000, Anti GFP (HRP conjugate) (Abcam) 1:10000.

Structural models and protein alignments

SWISS-MODEL (Swiss Institute of Bioinformatics) online software was used to generate a structural homology model of Kir7.1 based on the published crystal structure of Kir2.2 PDB 5KUK. Models were visualized using PyMol (Schrodinger). Protein alignments were performed with either Clustal Omega (EMBL-EBI) or Lasergene MegAlign software (DNASTAR) using the UniProt entry human sequences for Kir7.1(KNCJ13_O60928), Kir4.1 (KCNJ10_P78508), Kir5.1 (KCNJ15_Q99712), Kir3.1 (KCNJ3_P48549), Kir2.1 (KCNJ2_P63252), Kir 1.1 (KCNJ1_P48048).

Whole cell electrophysiology

Whole cell patch-clamp electrophysiology was performed as described in Raphemot et al 2014(Raphemot, Swale et al. 2014). Briefly, HEK-293T cells were transfected with Kir7.1-M125R encoding plasmids (0.5µg), MC4R plasmids (3.75 µg) or β2AR plasmids (3.75 µg) and EGFP-plasmid (0.05 µg) as a marker for transfections. The following day, cells were dissociated with trypsin and plated on poly-l-lysine coated glass coverslips. The plated cells were allowed to recover for 1 hour prior to experiments. The cover slips were placed in the recording chamber on an inverted microscope stage and perfused with a bath solution containing 135mM NaCl, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 5mM glucose, 10mM HEPES, pH 7.4. A Flaming-Brown P-1000 micropipette puller was used to pull electrodes with resistances between 2 and 3 MΩ. The pipettes were filled with 135 mM KCl, 2mM MgCl₂, 1mM EGTA, 10mM HEPES, pH 7.3. Transfected cells were identified by EGFP fluorescence, and voltage-clamp conditions were used to record whole cell currents. To obtain a current-voltage curve, cells were voltage clamped at a holding potential of -75mM, and then stepped to -150mV for 500ms before ramping to 150mV at a rate of 2.4mv/msec. Cells were superfused with 4mM BaCl₂ at the end of the experiment to fully block all Kir7.1 channels. This value was used to derive barium inhibitable current.

Single channel electrophysiology

For electrophysiology experiments, cells were plated onto 12 mm cover glass chips at 4×10^4 in 35 mm diameter culture dishes, transfected after 24 hours with 0.3 µg cDNA of wt Kir 7.1 or Kir 7.1^{N95Q}, and 0.05 µg of EGFP (to identify transfected cells) using X-tremeGENE9 DNA transfection Reagent (Roche Diagnostics, Indianapolis, IN). Channel activity was recorded 48-72

hours after transfection in the cell-attached configuration. Pipettes had resistances of 6-10 M Ω when filled with a solution of the following composition (mM): 135 NaCl, 5 KCl, 10 MgCl₂, 2 CaCl₂, 5 glucose, 10 HEPES, pH 7.4, with NaOH. Cells were bath-perfused with a high-K⁺ solution of the following composition (mM): 135 KCl, 2 MgCl₂, 2 CaCl₂, 5 glucose, 10 HEPES, pH 7.4, with KOH. The resting membrane voltage was assumed to be 0 mV. Currents were recorded using an Axopatch 200B amplifier, filtered at 2 kHz, sampled at 20 kHz using Digidata 1550 and saved using pCLAMP 10.4 (Axon Instruments). Data were analyzed offline using TAC 4.2 and TACFit 4.2 (Bruyton Corporation) software. Single-channel open and closed events were analyzed using the 50% threshold detection method and visually inspected before accepting the events. Single-channel openings occurred as bursts of one or more openings or cluster of bursts. Duration time and amplitude histograms were generated using TACFit 4.2 (Bruyton Corporation, Seattle, WA, USA). Single-channel amplitudes (*i*) were calculated by fitting all-point histograms with single- or multi-Gaussian curves. The difference between the fitted 'closed' and 'open' peaks was taken as *i*. Duration histograms were fitted with exponential components in the form: $\sum(a_i/\tau_i)\exp(-t/\tau_i)$, where *a* and τ represent the relative area and time constant of the *i* component, respectively, and *t* is the time. Numerical data were expressed as mean \pm S.E.M. Statistical analysis was performed using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla, CA). Statistical significance was taken as $p < 0.05$ using unpaired two-tailed Student's *t* test.

ER Stress test

Cells were transiently transfected as previously described with an additional mock transfected control. The control cells were treated with BFA/Monesin (0.5x) for 12 hours to induce the unfolded protein response. Western blot analysis was performed as previously described, and

primary antibodies against CHOP (CST 1:1000), and the spliced isoform of XBP1s (CST 1:1000), with anti-rabbit secondaries (HRP conjugate 1:10,000) were used to detect ER stress markers. Expression of each marker was compared to the BFA/Monesin control and Kir7.1 only expressing cells, versus co-expression with GPCRs.

Co-immunoprecipitation

HEK293T cells were co-transfected with Kir7.1-3xFLAG and 3HA-B2AR and crosslinked with 1mM DSP dithiobis(succinimidyl propionate (pierce) for 30 minutes. Samples were quenched with 20mM Tris HCL for 20 minutes prior to harvesting in lysis buffer. Protein lysate was incubated with either anti-Flag M2 antibody (sigma) or anti HA (12CA5) (Vanderbilt antibody and protein resource VAPR) overnight with constant rotation at 4C. Protein G magnetic beads (Biorad) were added to the overnight lysate antibody sample with further rotation for an additional hour. A magnetic rack was used to capture the bead-antibody protein complex, followed by three washes with modified RIPA buffer. Protein was eluted from the beads with 2X LDS with 200mM DTT buffer, by heating for 10 minutes at 55°C. Eluted samples were analyzed by western blot analysis. Un-transfected protein samples, or samples expressing either Kir7.1-3xFlag or 3xHA-MC4R only were also subject to IP with anti-Flag M2 antibody (sigma) and anti HA (12CA5) to rule out non-specific detection of the protein tags.

APPENDIX

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“ 60 YEARS OF POMC: Regulation of feeding and energy homeostasis by α -MSH”

Anderson, E. J. P., I. Çakir, S. J. Carrington, R. D. Cone, M. Ghamari-Langroudi, T. Gillyard, L. E. Gimenez and M. J. Litt (2016) Journal of molecular endocrinology **56**(4): T157-T174

Kir7.1 and other ion channels

Based on the finding that AgRP couples MC4R to the pertussis toxin-sensitive Gi/o inhibitory protein in the hypothalamic GT1–7 cell line (Buch, Heling et al. 2009), it was suggested that MC4R might be coupling to G protein inwardly rectifying potassium channels (GIRKs). This result highlights the ability of MC4R to signal through different G proteins with opposing actions. One is the canonical (for MC4R) Gs α adenylyl cyclase stimulatory action activated by α -MSH vs Gi/o inhibitory protein-dependent pathways promoted by AgRP. The ability to signal through Gi/o led to the hypothesis that MC4R could potentially couple to GIRKs, known to be activated by G $\beta\gamma$ binding following release from Gi heterotrimers (Lei, Jones et al. 2000). Physiologically relevant data on the mechanism of α -MSH and AgRP signaling through native MC4R derives from the use of electrophysiological slice preparations from mice in which PVH MC4R neurons have been transgenically labeled with GFP (Ghamari-Langroudi, Vella et al. 2010, Ghamari-Langroudi, Srisai et al. 2011). In this preparation, α -MSH depolarizes and AgRP hyperpolarizes MC4R neurons when added to the bath. Notably, by using this experimental setup in the presence of G protein inhibitors such as GDP β S and gallein, the role of GIRKs in α -MSH induced depolarization in MC4R-positive PVH neurons was ruled out.

Moreover, G α and cAMP signaling were also ruled out, when examined as a potential signaling pathway mediating α -MSH-induced depolarization (Ghamari-Langroudi, Digby et al. 2015). Current-voltage analyses demonstrated that the conductance involved in α -MSH-induced depolarization was a potassium inward rectifier channel (Kir), and a specific subunit, Kir7.1, was identified using a panel of specific Kir channel blockers.

Also of interest is that AgRP, the endogenous inverse agonist for cAMP responses from MC4R, hyperpolarizes PVH neurons (Ghamari-Langroudi, Digby et al. 2015). AgRP augmented membrane conductance, suggesting increased potassium channel opening and surface density, leading to hyperpolarization. Cell transfection studies reinforced the finding that AgRP couples the MC4R to Kir7.1 and regulate its opening. The molecular mechanism by which AgRP mediates this effect currently remains unknown and will require further studies to be definitively clarified. Other inward rectifiers such as Kir2.3 and Kir4.1 channels did not couple to MC4R in independent experiments using cultured cells. As Kir channels form homo- and heterotetramers, it remains to be determined whether MC4R modulates homotetramers of Kir7.1 or heterotetramers with other Kir channel subunits. Kir7.1 modulation by MC4R also presented new pharmacological paradigms for the receptor, where some ligands have been found to favor the G α vs the Kir7.1 pathway. (Ghamari-Langroudi, Digby et al. 2015) reported changes in potency for well-characterized tool compounds of MC4R, where the MSH analog MC4-NN2-0453, with an EC₅₀ of 4.9×10^{-9} M for intracellular cAMP accumulation assays, was found to have an increased potency of 4.5×10^{-9} M in a thallium flux-based assay used to measure Kir7.1 coupling. Taken together, these findings reveal a novel MC4R signaling pathway, and the potential for the creation of biased ligands that favor the ion channel direct interaction/activation paradigm over classical G protein signaling,

making this receptor one of only a handful of GPCRs that were shown to couple directly to ion channels (Zhang, Li et al. 2014).

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