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G-protein coupled receptor kinases in inflammation and disease

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Abstract

G-protein coupled receptor kinases (GRKs) are serine/threonine protein kinases originally discovered for their role in G-protein coupled receptor (GPCR) phosphorylation. Recent studies have demonstrated a much broader function for this kinase family including phosphorylation of cytosolic substrates involved in cell signaling pathways stimulated by GPCRs as well as non-GPCRs. In addition, GRKs modulate signaling via phosphorylation-independent functions. Because of these various biochemical functions, GRKs have been shown to affect critical physiological and pathophysiological processes and thus are considered as drug targets in diseases such as heart failure. Role of GRKs in inflammation and inflammatory diseases is an evolving area of research and several studies including work from our lab in the recent years have demonstrated critical role of GRKs in the immune system. In this review we discuss the classical and the newly emerging functions of GRKs in the immune system and their role in inflammation and disease processes.

1.1: Introduction

Cells are exposed to myriad of extracellular agents including hormones to which the cells have developed sophisticated mechanisms for receiving, processing and transmitting signals. Receptors play a critical role in receiving these signals, and are present both on the plasma membrane and inside the cells. Among these receptors, G-protein coupled receptors (GPCRs) form the largest family of membrane receptors that are encoded by ~950 genes[1]. These GPCRs are characterized by their seven transmembrane domain and detect a range of extracellular signals including neurotransmitters, chemoattractants, lipids, peptides, hormones, light and odors. Transmission of signals via GPCR activation modulates a variety of physiological processes including sense of vision, olfaction, hormonal signal transduction, cellular proliferation, differentiation, and cell survival. Because of the multitude of signals received by these GPCRs, these receptors are now direct drug targets for ~50% of the currently used therapeutics[2]. Classical GPCR activation by agonist binding causes conformational change in the receptor which results in the activation of the heterotrimeric GTP-binding proteins (G-proteins)[3]. G-proteins are a complex of subunits composed of α subunit (Gα- encoded by 16 genes) and βγ dimers (Gβ encoded by 5 genes and Gγ encoded by 12 genes) [4]. Exchange of GTP for GDP in Gα leads to dissociation of Gα from Gβγ. However, there is also evidence that in some cases the heterotrimers may not

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fully dissociate[5]. Instead, they may undergo structural rearrangement following GPCR activation. Subsequent to GPCR activation and exchange of GTP for GDP in Gα, Gα-GTP and Gβγ activate a number of effector proteins leading to various biological outcomes (Fig 1). The intrinsic GTPase activity in Gα subunit causes GTP hydrolysis, and formation of Gα-GDP leading to re-association of Gαβγ trimer. For a comprehensive review of G-protein activation please see other reviews[6, 78].

One of the fundamental mechanisms in the regulation of GPCR signaling is the ability of the receptor to "shut down" upon continuous stimulation. This phenomenon called "desensitization" is mediated by two protein families: G-protein coupled receptor kinases (GRKs) and arrestins. Members of these two protein families play critical roles in desensitization of most GPCRs. GRKs specifically phosphorylate agonist occupied GPCRs and this results in arrestin translocation and high affinity binding to the phosphorylated receptor. Arrestin binding interdicts GPCR and G-protein binding and this event functionally uncouples GPCRs from their cognate G-proteins thereby terminating G-protein activation [9, 10].

In addition to the classical desensitization functions, studies within the last decade clearly emphasize functions of GRKs and arrestins that are distinct from this canonical role. It is now clear that GRKs (and arrestins) have GPCR-dependent but G-protein independent functions in cell signaling and biology. Importantly, GRKs and arrestins have also been shown to modulate GPCR-independent functions in physiological processes. In recent years, this role of GRKs has especially become apparent in the context of inflammation and inflammatory diseases. In this review, we discuss the emerging themes of GRK functions especially those of non-visual GRKs, in both GPCR-dependent and –independent functions relevant to inflammatory processes.

1.2: The G-protein coupled receptor kinase family

During 1970s and mid-1980s, agonist induced dampening of G protein-mediated signaling was discovered for rhodopsin and β2-adrenergic receptor. The enzyme phosphorylating rhodopsin receptor (which controls vision) was identified in the late 1970s and aptly termed as "Rhodopsin kinase" (aka GRK1)[11]. Few years later, a novel kinase was demonstrated to phosphorylate β2-adrenergic receptor and dampen G protein-mediated signaling[12]. This kinase was initially named as β-adrenergic receptor kinase or βARK (aka βARK1, GRK2). Simultaneously, cloning of the mammalian β-adrenergic receptor revealed a sequence similarity with rhodopsin receptor and this led to the recognition of GPCR family, with rhodopsin as its founding member[13]. Another protein (termed as S-antigen) discovered initially for its role in allergic uveitis[14], was found to associate with rhodopsin and dampen G-protein mediated retinal signaling[15, 16]. In 1986, with the discovery of this "arresting" function in retinal signaling, this protein was renamed as "arrestin". Further, functional characterization of GRKs and arrestin established the idea of two-step inactivation process of desensitization of GPCRs. Meanwhile, other members of the GRK family were identified: GRK3 (aka βARK2) [17], GRK4 [18], GRK5 [19], GRK6 [20] and GRK7[21, 22].

GRKs are grouped broadly into visual and non-visual GRKs. Expression of the visual GRKs (GRK1 and GRK7) is restricted to the eyes and pineal gland. The non-visual GRKs are further grouped into two sub-groups: GRK2-like [GRK2 and GRK3, otherwise known as *β*ARK1 and 2 (*β*-adrenergic receptor kinases 1 and 2)] and GRK4-like (GRK4, GRK5 and GRK6) based on their structural similarity[23]. Non-visual GRKs are widely distributed throughout the body with the exception of GRK4, which is found restricted in the testis and proximal tubule of the kidney. Therefore, most GPCRs in the body are regulated by at least one of the four GRKs (GRK2, GRK3, GRK5 and GRK6). Given that there are hundreds of GPCRs in the mammalian system, each GRK must regulate more than one GPCR, thus increasing the complexity of our understanding the role of GRKs.

All GRKs are multi-domain proteins, sharing a 25 residue N-terminal region (unique for GRKs) followed by a regulator of G-protein signaling homology domain (RH) and a catalytic serine/threonine protein kinase domain responsible for phosphorylating substrates (Figure 2). The N-terminal basic region in the GRK4-like family (GRK4-6) aid in membrane translocation. However, the C-terminal domain is the most essential domain for targeting the GRKs to the plasma membrane. GRK1 and 7 are membrane associated by virtue of their short farnesylated C-termini. The C-termini of GRK2-like kinases are longer than that of GRK4-like kinases and contain a 125 amino acid pleckstrin homology (PH) domain. PH domain has binding sites for phosphatidylinositol bisphosphate (PIP2) and Gβγ. GRK4 and GRK6 are thought to be membrane associated via their palmitoylated residues within the last 15-20 amino acids of C-terminus and also by their N-terminal basic region. GRK5 is also predominantly membrane bound, through the poly basic regions found in both C- and N- termini[24]. Interestingly, GRK5 also contains a nuclear localization signal and has been shown to accumulate inside the nucleus. Indeed, GRK5 can phosphorylate class II histone deacetylase and therefore mediate gene transcription in cardiomyocytes[25]. Interestingly, GRK6A, one of the three splice variants of GRK6, has also been detected inside nucleus; however, its physiological role is yet to be ascertained[26]. In addition to these differences, recent studies have also shown that the GRK2/3 and GRK5/6 family members differ in their ability to phosphorylate inactive GPCRs. Although it was originally assumed that all GRKs phosphorylate only active GPCRs, GRK5 and 6 were shown to phosphorylate β2-adrenergic and M2 muscarinic receptors even in their inactive state[27].

1.3: Regulation and Activation of GRKs

Activity of GRKs is regulated by both protein-protein interactions and phosphorylation events. Gβγ subunits were one of the earliest known proteins known to interact with and activate GRKs[28]. In addition, lipids can bind and activate GRKs. Interestingly GRK2 family members are activated by phospholipids (PIP2) binding to C-terminal PH domain whereas; GRK4-like family members are activated by PIP2 binding to N-terminal polybasic regions[29]. Furthermore, calmodulin, caveolin-1, and actin are also known to affect GRK activity by direct binding[30, 31].

Phosphorylation can lead to either activation or deactivation of GRKs depending on which kinase phosphorylates GRKs or whether GRKs undergo autophosphorylation. A decrease in kinase activity of the respective GRKs has been observed when visual GRKs are

phosphorylated by protein kinase-A (PKA) and GRK5 by protein kinase C (PKC)[32,33]. In contrast, GRK2 phosphorylation by PKC enhances its kinase activity[34]. In addition to phosphorylation, GRK2 can also be s-nitrosylated by nitric oxide synthase, which leads to inhibition of its activity[35]. These findings suggest that multiple pathways are involved in regulating the activities of GRKs.

Recent studies looking at the crystallographic structures of GRK1, GRK2 and GRK6 have provided key information on how these GRKs phosphorylate receptors[36]. These studies revealed GRKs in a closed conformation in which the conserved N-terminal 18-20 residues form the alpha (αN) helix and interact with the kinase domain. Formation of αN helix stabilizes the closed (active) conformation of the kinase domain leading to allosteric activation and favoring catalysis by kinase domain. Furthermore, the αN helix is proposed to act as docking site for the activated receptors and removal of αN helix abolishes GPCR phosphorylation, suggesting αN helix formation is indeed the primary step in the activation of GRKs.

1.4: Animal models of GRK deficiency

Phenotypes of GRK knockout mice have enabled researchers to identify the various pathophysiological roles of GRKs. In some cases, phenotypes were less obvious likely due to compensation by other GRKs. The most striking phenotype was that of embryonic lethality in GRK2 homozygous knockout due to defective cardiac development[37]. Using targeted knockouts and heterozygous mice GRK2 has been shown to be important in the heart development, lymphocyte chemotaxis, experimental autoimmune encephalomyelitis, sepsis, atherosclerosis etc. Closer examination of other GRK knockout mice revealed distinct phenotypes- GRK3 in olfaction[38]; GRK5 in cholinergic responses and inflammation; GRK6 in chemotaxis, behavioral responses, locomotor stimulating effect of cocaine etc.[39]. A detailed summary of these phenotypes is listed in Table 1.

In addition to knockout mice, transgenic over-expression of GRKs has also revealed important functions for GRKs[40, 41]. Thus, research from over the last decade demonstrates multiple functions of GRKs in different organ systems. However the role of GRKs in immune system is only beginning to be understood. In this review, we will focus mainly on our recent understanding of GRKs in the regulation of immune system, in particular their effects on inflammatory signaling pathways and in inflammatory diseases.

REGULATION OF INFLAMMATORY SIGNALING PATHWAYS BY GRKs

GRKs in Nuclear Factor κ-B signaling

Nuclear factor κ-B (NFκB) signaling pathway is intricately tied with many inflammatory processes and thus, regulatory molecules in NFκB signaling pathway are potential therapeutic targets in a number of inflammatory diseases. Under unstimulated conditions, NFκB transcription factors (p65 (RelA), p50, RelB, cRel and p52) are sequestered in the cytoplasm in complex with members of the IκB family of proteins (IκBα, IκBβ, Iκβε, p105 (NFκB1), and p100 (NFκB2)). Upon stimulation, IκB is phosphorylated by IκB kinase (IKK) complex. The phosphorylated IκB then undergoes ubiquitination and subsequent

degradation, releasing the NFκB transcription factor, which then translocates into the nucleus to modulate gene transcription. GRKs, in particular GRK2 and GRK5 have been reported to modulate NFκB signaling pathway in immune and non-immune cells. In earlier studies, we showed that GRK5 directly interacts with NFκB p105 (one of the IκB members) and inhibits TLR4-induced IKKβ-mediated phosphorylation of p105[42]. Although GRK5 is able to phosphorylate p105, identity of the amino acid residues is still under investigation. Subsequent to the discovery of GRK5 regulation of p105, Iaccarino et al[43] reported that GRK5 binding to IκBα (another IκB member) stabilizes IκBα and facilitates nuclear accumulation of IκBα by masking the nuclear export signal (NES) sequence. Nuclear accumulation of IκBα led to decreased NFκB activation in endothelial cells. This GRK5 regulation of IκBα was shown to be dependent on the RH domain but independent of its catalytic activity.

Later studies however underscored further complexity in the regulation of NFκB pathway by GRK5. Studies by Valanne *et al* demonstrated that the role of GRK5 in NFκB signaling has an evolutionarily conserved significance and that GRK5 is a critical *mediator* of NFκB signaling in different models (and species) including Drosophila, Zebra fish and human cell lines[44]. In their studies GRK5 was shown to be required for normal microbial resistance *in vivo* in Zebrafish and Drosophila[44]. Later studies from our group demonstrated that GRK5 is a non-canonical IκBα kinase and that it phosphorylates Ser32/36- same sites phosphorylated by IKKβ, the canonical IκBα kinase[45]. Consistent with these biochemical findings, levels of cytokines and chemokines were largely attenuated in GRK5 knockout mice compared to the wild type mice in endotoxemia model[46]. In addition, IκBα phosphorylation and p65 nuclear translocation were significantly reduced in LPS treated GRK5 deficient peritoneal macrophages. Interestingly, using a different GRK5 knockout mice generated by the Lefkowitz group, Wu *et al*[47] found that endothelial GRK5 indeed stabilized IκBα similar to earlier studies by Iaccarino et al[48]. Also, unlike our studies in macrophages, Wu *et al* did not find any role for GRK5 in IκBα phosphorylation and p65 translocation. It is yet unclear if these contrasting results are due to different cell types being studied, different knockouts used (complete deletion of GRK5 gene versus part of the gene), or background of the mice. It should be noted however, that a recent study by Islam *et al* using GRK5 knockout mice from Lefkowitz group found that GRK5 indeed positively regulates NFκB pathway in cardiomyocytes[49]. In earlier studies, Koch's lab had shown that GRK5 expression itself is regulated by NFκB pathway suggesting a positive feedback loop wherein GRK5 mediates NFκB pathway and NFκB signaling increases GRK5 expression[50]. In primary peritoneal macrophages however, TLR ligands down regulate GRK5 expression suggesting that both regulation of GRK5 expression and its role in NFκB pathway are distinct in different cell types. Given that NFκB signaling is a double-edged sword, the physiological relevance of GRK5 regulation of the NFκB pathway can only be deduced from *in vivo* disease models (discussed later). Interestingly, while this review was in preparation, Ohba *et al* showed that GRK6 is also able to phosphorylate IκBα at Ser32/36. Similar to the role of GRK5, TNFα-induced inflammation in peritoneal macrophages was shown to be dependent on GRK6. Importantly, TNFα was shown to induce a conformational change in GRK6[51].

Compared to GRK5, GRK2 has also been shown to interact with IκBα and p105. Unlike GRK5 (and 6) GRK2 phosphorylates IκBα at very low stoichiometry[52]. Similar to GRK5 however, GRK2 negatively regulates p105 signaling in primary peritoneal macrophages, *via* interaction with p105[53]. Interestingly, TLR ligands enhance GRK2 expression in primary macrophages[54]. In addition, immune cells from septic patients exhibit high GRK2 levels[55] suggesting that GRK2 levels and the associated signaling pathways have potential clinical relevance in inflammatory diseases.

GRKs in Mitogen Activated Protein Kinase (MAPK) signaling

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that mediate fundamental biological processes and cellular responses to extracellular signals. Typically, activation of MAPKs lead to phosphorylation events that culminate in translocation of transcription factors from the cytoplasm to the nucleus leading to altered gene expression. Three major groups of distinctly regulated MAP kinase cascades are known to lead to transcription of various genes: ERK1/2, JNK, and p38 MAP kinase. ERK is activated by MAP kinase kinase1 (MKK1) and MKK2, JNK by MKK4 and MKK7, and p38 MAP kinase by MKK3, MKK4, and MKK6. Upon activation of the MAP kinases, transcription factors present in the cytoplasm or nucleus are phosphorylated and activated, leading to expression of target genes resulting in biological responses.

A wide range of extracellular signals including LPS and TNFα can induce the ERK1/2 pathway. Activation of ERK1/2 pathway leads to the induction of various inflammatory mediators (e.g. TNFα, IL-1, IL-8 and PGE2). Both GRK2 and 5 can negatively regulate LPS-induced ERK pathway in macrophages[42, 53]. Also overexpression of GRK5 and/or GRK6 was found to enhance β-arrestin2-mediated ERK1/2 activation, whereas overexpression of GRK2 and/or GRK3 abolished β-arrestin2-mediated ERK1/2 activation[56]. These effects were observed with activation of β2 adrenergic receptor, cannabinoid receptor 2[57], Angiotensin 1A receptor[43,58] and Insulin like growth factor 1 receptor[59].

The p38 MAP kinase pathway shares many similarities with the other MAP kinase cascades, and is also associated with inflammation, cell growth, cell differentiation, and cell death. A number of pathogenic stimuli, including LPS, staphylococcal peptidoglycan and enterotoxin B, echovirus 1 and herpes simplex virus 1 activate p38 through different toll like receptors[60-62]. The main biological response of p38 activation in the immune system involves the expression and production of inflammatory mediators to initiate leucocyte recruitment and activation. P38 MAPK mediates expression of many genes involved in inflammation, such as TNFα, IL-1β, IL-6, IL-8, cyclooxygenase-2, as well as collagenase-1, and -3[63]. Inhibition of p38 MAPK with SB203580 reduces pro-inflammatory cytokine production in monocyte/macrophages, neutrophils, and T lymphocytes[64]. Interestingly, GRK2 and p38 have bidirectional functional roles. Whereas GRK2 inhibits p38 function by directly phosphorylating it, p38 blocks GRK2 mediated GPCR desensitization[6566]. GRK2 phosphorylates p38 MAPK at Thr123 and interferes with the ability of p38 to bind to MKK6 and therefore prevents p38 activation[65]. In addition, modulating GRK2 levels alters activation of p38 and its-dependent processes such as differentiation of adipocytes and cytokine production. Consistent with this role, $GRK2^{+/-}$ macrophages[65] and microglial

cells[67] have increased p38 activation and produce increased amounts of TNFα in response to LPS and this results in accelerated brain damage during hypoxic ischemic injury in neonatal mice[67]. In contrast, GRK2 silencing decreases cytokine production (IL-6 and IL-13) in a p38-dependent manner during antigen-induced mast cell degranulation[68]. Interestingly, GRK2 and GRK5 can phosphorylate a constitutively active, virally encoded GPCR (US28) and inhibit its activation but simultaneously mediate p38 MAPK activation[69]. Both GRK2 and GRK6 also regulate cytokine-induced pain in a p38 MAPK-dependent manner. These kinases reduce neuronal responsiveness to cytokines such as IL-1β and TNFα, by downregulating p38 activation thereby reducing cytokine-induced hyperalgesia[70, 71].

Compared to GRK2 regulation of p38 activity, p38 directly phosphorylates GRK2 at Ser670 and inhibits GRK2 translocation to the membrane, thereby preventing GRK2-initiated internalization and desensitization of CCR2 in response to MCP-1[72]. In addition, p38 inhibits GRK2-mediated desensitization by acting as a non-canonical GRK for the FPR1 (Formyl Peptide Receptor1) and facilitating neutrophil chemotaxis[66].

JNKs are activated by mitogens as well as by a variety of environmental stresses (heat shock, ionizing radiation, and oxidants), genotoxins (topoisomerase inhibitors and alkylating agents), ischemic reperfusion injury, mechanical shear stress, vasoactive peptides, proinflammatory cytokines and PAMPs/DAMPs[73-77]. JNK induces transcription of AP-1, c-Jun, ATF-2, and ELK-1, all of which are important mediators of inflammatory gene transcription[75]. JNK activation of AP-1 is important for synthesis of TNFα, as well as proliferation and differentiation of lymphocytes and hence plays a vital role in immune system[77, 78]. Role of GRKs in JNK signaling particularly related to the immune system is not well characterized. However, studies with transgenic mice overexpressing cardiac specific GRK5 and constitutively active mutant of α1B-adrenergic receptor showed attenuation of JNK activation compared to controls. GRK5 also had variable effects on α_{1B}AR signaling, and the complexity of GRK5 regulation in *in vivo* α_{1B}AR signaling remains to be fully elucidated[79].

Together, these studies suggest that while some aspects GRK regulation of the MAPK pathways have been explored in the immune system, most of these studies are focused more on GRK2 and some on GRK5. Role of GRK3 and 6 in these pathways remain less understood. Moreover, it remains to be seen if regulation of MAPK pathways by GRKs is therapeutically targetable in inflammatory diseases.

Regulation of immune processes by GRKs

GRKs in Immune cell migration—Chemotaxis is an important function, which enables immune cells to arrive at the site of inflammation. Cells producing chemokines act on chemokine receptors (mostly GPCRs), and initiate chemotactic response[80]. The chemotactic response depends on the amount of chemokines produced and their gradient and also on the expression levels of their receptors. Chemotaxis also depends on the integrated modulation of different steps of the chemotactic processes (receptor sensing, cell polarization, membrane protrusion, adhesion/de-adhesion cycles) in a given cell type and in response to specific stimuli[81]. Chemokine receptors being GPCRs also undergo desensitization upon continuous presence of stimuli. Therefore it is not surprising that GRKs are critical

regulators of chemotaxis. Intriguingly, GRK2, -3, -5 and -6 are expressed at high levels in immune cells suggesting modulation of these GRKs during disease might change the outcome or progression of the disease via modulation of immune cell chemotaxis.

Of the various GRKs, GRK2 is widely studied and better characterized in terms of chemotaxis. Studies have shown that GRK2 regulation of cell migration is complex- it is both cell type and stimulus-dependent[82, 83]. In most cell types GRK2 negatively regulates chemotactic responses consistent with its canonical negative regulatory role in GPCR signaling [72, 84, 85]. Cell lines transfected with GRK2 and chemokine receptors, show increased agonist-induced phosphorylation and/or desensitization of chemokine receptors such as CCR2b[86], CCR5[87] and CXCR1[88]. Consistent with its negative regulatory role reduced GRK2 levels in T-lymphocytes increase chemotactic response to CCL3, 4 and 5 which act through chemokine receptors CCR1 and CCR5[89]. In addition, myeloid specific GRK2 knock out and LDL receptor knock out chimeric mice exhibit increased mobilization of macrophages to inflammatory sites and have increased circulating neutrophils, conforming to the desensitizing effect of GRK2 on chemokine receptors[90]. Moreover, neutrophils obtained from patients with different disease conditions such as malaria[91] and sepsis[55] reveal increased GRK2 levels associated with decreased CXCR2 expression and reduced response to IL-8. This increased GRK2 expression might be deleterious from the host perspective since this could reduce appropriate chemotactic response and eventually the ability of the host to contain the pathogen. On that note, IL-33 reverses sepsis-induced expression of GRK2, resulting in increased CXCR2 expression in neutrophils leading to increased chemotaxis of neutrophils, increased bacterial clearance and enhanced survival in mice[92]. In contrast to these studies, GRK2 positively mediates chemotactic responses in few other cell types[93]. These roles of GRK2 might depend on the cell type and its polarization state. In polarized cells such as epithelial cells[93], GRK2 mediates chemotaxis, whereas in the less polarized cells such as immune cells[89], GRK2 does the opposite. Interestingly, positive regulation of chemotactic responses by GRK2 does not require its catalytic activity thus suggesting protein-protein interaction being a potential molecular mechanism[94]. In keeping with this notion, membrane-targeted kinase mutant strongly enhances cell motility, and GRK2 interacts with GIT1 (GRK2 interacting factor), and is present at the leading edge of polarized/migrating epithelial cells in wound-healing assays[94]. Also, this transient association of GRK2 with GIT1 is critical for proper ERK1/2 activation and efficient cell migration. GRK2 has also been shown to directly phosphorylate histone deacetylase 6 (HDAC6), a cytoplasmic histone deacetylase responsible for deacetylation of tubulin and other substrates involved in cell migration[95]. Furthermore, GRK2 also phosphorylates ERM proteins ezrin and radixin, which contribute to the F-actin polymerization-dependent motility[96, 97]. These novel roles of GRK2 in cell migration might shed light in comprehending the non-canonical role of GRK2 in cell motility.

Similar to GRK2, GRK5 has also been shown to regulate GPCRs that are critical in chemotaxis in a canonical as well as non-canonical manner. However, unlike GRK2, GRK5 has been shown to regulate cell migration in very few cell types. In monocytes, GRK5 regulates cell migration by modulating CC chemokine receptor-2 (CCR2), a GPCR for monocyte chemoattractant protein-1[47]. GRK5 also modulates monocyte chemotaxis in a non-GPCR dependent fashion by regulating the colony-stimulating factor-1 receptor

(CSF-1R), a receptor tyrosine kinase[98]. Furthermore, GRK5 was reported to attenuate atherosclerosis by desensitizing CCR2 and inhibiting migration of monocytes[47]. GRK5 was also shown to regulate CXCR4 (CXC chemokine receptor-4) desensitization via phosphorylation of HIP (HSP70 interacting protein) in an *in vitro* system[9]. However, *in vivo* evidence of GRK5-mediated desensitization of CXCR4 is currently lacking. In our studies using a clinically relevant model of polymicrobial septic peritonitis, there was no evidence for a role for GRK5 in immune chemotaxis[10]. Similar findings have been reported for arthritis[99]. GRK5 has been shown to regulate prostate cancer cell migration and invasion by forming a complex with moesin (ERM- ezrin-radixin-moesin) proteins. In this study, authors found that GRK5 phosphorylates moesin principally on Thr-66 residue, thereby regulating cellular distribution of moesin[100]. Interestingly, a recent study demonstrated that GRK5 can function as an actin-bundling scaffold and promote neuronal filopodial formation and neurite outgrowth [101].

GRK6 has been shown to regulate chemokine receptors CXCR2[88], CXCR4[102] and LTB4[103]. By desensitizing these receptors, GRK6 modulates neutrophil and lymphocyte recruitment *in vivo* in different disease models[99, 104, 105]. In epithelial cells, a functional screening identified GRK6 as a critical mediator in integrin-mediated cell adhesion and migration of tumor cells[106], and GRK6 deficiency also appears to promote CXCR2-mediated tumor progression and metastasis in a lung carcinoma model[107]. These roles of GRK6 are mostly related to its canonical role in GPCR desensitization. Evidence for GRK6 in non-canonical role of immune cell chemotaxis is currently lacking.

Together, these studies reveal critical functions of GRKs in immune cell chemotaxis and therefore suggest that if specific GRKs could be selectively modulated by small molecule compounds, that could have a potential therapeutic effect in disease processes.

GRKs in cell apoptosis—Apoptosis or programmed cell death regulates development, selection, and maturation of immune cells at various stages of their life cycle. Hence, normal rate of apoptosis is critical in immune system development. Inappropriate apoptosis (either too little or too much) is a factor in many human conditions including sepsis, neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. In addition to the role of apoptosis in development of immune system, apoptotic bodies/cells can alter the course of the inflammation. Apoptotic cells are usually taken up by macrophages and dendritic cells (DC). In response to the uptake, macrophages induce production and release of immunosuppressive cytokines such as IL-10, TGF-β, prostaglandin E2 and PAF[108] and suppress production of proinflammatory cytokines IL-1β, IL-6, IL-12 and TNFα [109]. Similarly, ingestion of apoptotic cells by DCs also results in suppression of IL-12 and IFN-γ expression, upregulation of co-inhibitory molecules and production of anti-inflammatory cytokines[110]. Thus, apoptotic cells have significant impact on the function of these phagocytes and that in turn modulates inflammatory disease pathogenesis.

Consistent with their broad biological functions, GRKs have also been reported to play critical role in apoptosis; however, there have been only a few studies looking at the role of GRKs in immune cell apoptosis especially in *in vivo* disease models. GRK2 overexpression

was shown to increase caspase-3 levels and induce increased cardiomyocyte apoptosis following ischemia/reperfusion injury in myocardium. Conversely, GRK2 inhibition reduced apoptosis via increased NOS activity, NO production and AKT levels in cardiomyocytes[111]. These data suggest positive regulatory role of GRK2 in apoptosis [112]. Interestingly, GRK2 levels were increased in apoptotic lymphocytes obtained from heart failure patients. Even though the implications of this is not yet clear, it is possible that increased GRK2 levels may drive more lymphocyte apoptosis.

Chen *et al* demonstrated that GRK5 is able to phosphorylate p53 and regulate irradiation-induced apoptosis[113]. p53 is crucial in determining the cellular response to stress, not only by inducing apoptosis but by also having a role in growth arrest. Induction of p53-dependent apoptosis occurs *via* extrinsic or intrinsic pathways depending on apoptotic signals and converges on activation of caspases. GRK5 phosphorylates p53 *in vitro* and *in vivo* at Thr-55, which results in reduced p53 levels. Treatment with the proteasome inhibitor, MG132, prevents the reduction in p53, thus confirming its degradation is *via* the proteasomes. GRK5 knockout mice display tissue-wide upregulation of p53 implying that GRK5 negatively regulates p53 *in vivo.* GRK5-mediated p53 degradation directly affects apoptosis, as knockdown of GRK5 in the osteosarcoma cell line, U2OS, increases apoptosis by 40% following cisplatin treatment. Apoptosis of Saos-2 cells, which are p53 null, were unaffected following GRK5 knockdown, suggesting that GRK5 induces apoptosis *via* p53[113]. In contrast, our studies suggest that GRK5 positively regulates thymocyte apoptosis *in vivo* in polymicrobial sepsis[10]. Specifically, lack of GRK5 causes decreased apoptosis of $CD4^+CD8^+$cells in the thymus. Additionally, GRK5 has been shown to negatively regulate Bcl-2 transcription (anti apoptotic protein) in SHSY5Y cells and this is predicted to increase cell death[114]. Together, these results suggest that the role of GRK5 in apoptosis may be related to multiple factors including the type of cell and context (*in vivo* versus *in vitro*).

Recently, Nakaya et al reported that GRK6 is able to regulate clearance of apoptotic cells[115]. GRK6 was shown to cooperate with GIT1 to activate Rac1, which promotes apoptotic engulfment. GRK6 was critical in removing apoptotic B cells by splenic white pulp macrophages and removing senescent red blood cells by splenic red pulp macrophages. GRK6-deficient mice were also shown to have increased iron stores in splenic red pulp in which $F4/80^+$ macrophages are responsible for senescent red blood cell clearance. As a consequence, GRK6-deficient mice were shown to develop autoimmune disease.

In summary, it is clear that GRKs can regulate chemotaxis, inflammatory signaling and apoptotic pathways. Because dysregulation of any one of these functions have greater impact in altering the course of many diseases, a number of studies have examined the role of specific GRKs in various disease processes, especially inflammatory diseases using genetically modified mouse models.

Role of GRKs in Neurodegenerative and autoimmune diseases

GRKs are implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD)[116], Multiple sclerosis (MS)[117] and Parkinson's disease[118]. GRK2 was shown to serve as a marker for early hypoperfusion induced brain damage which is associated with mitochondrial damage found in AD patients[119]. GRK2 levels in the brain are increased

during early stages of damage in aged human and in AD patients (observed postmortem)[119]. GRK2 via p38-dependent TNFα production exacerbates brain damage during hypoxic ischemic injury[67]. GRK2 was also shown to regulate metabotropic glutamate receptor function and expression, which has been implicated in AD and MS pathogenesis[120, 121]. Also, studies have shown GRK2 downregulation following prolonged inflammation sensitizes human and rodent neurons to excitotoxic neurodegeneration via over-activation of group I mGluRs[121].

GRKs are also known to play a role in inflammatory hyperalgesia. GRK2 deficient heterozygous mice suffer from chronic hyperalgesia due to continued microglial activation via p38-dependent TNFα production[70, 122] as well as via prolongation of PGE_2-mediated pathways. The latter involves interaction with EPAC1 (Exchange protein directly activated by cAMP), activation of PKCε- and ERK-dependent signaling pathways[123-125]. Interestingly, even a transient decrease in GRK2 levels (by intrathecal injection of *Grk2* antisense oligodeoxynucleotides (asODNs)) is sufficient to produce a long-lasting neuroplastic change in nociceptor function leading to chronic pain[126].

GRK5 has been shown to regulate desensitization of muscarinic receptors selectively with a preference for M2 and M4 receptors[127]. These receptors are present in the presynaptic cells and negatively regulate acetylcholine (ACh) release in the hippocampal memory circuits. Increased presynaptic cholinergic activity decreases ACh release and decreases post-synaptic muscarinic M1 activity. Interestingly, M1 signaling has been shown to inhibit β-amyloidogenic APP processing and decreased β-amyloid accumulation[128]. This might explain the increased incidence of AD like pathology observed in GRK5 deficient mice.

Recent studies have demonstrated a physiological role for GRK6 in regulating apoptotic cell clearance in splenic red pulp and mice deficient in GRK6 develop autoimmune disease[115]. As discussed in the previous section, GRK6 mediates macrophage-dependent apoptotic cell clearance by phosphorylation of radixin and moesin, which are essential in membrane skeleton re-organization. Additionally, GRK6 has also been shown to modulate arthritis and colitis by regulating infiltration of immune cells[99, 105]. In DSS induced colitis model, GRK6 deficient mice produced more keratinocyte chemokine (KC), causing increased infiltration of immune cells and enhanced severity. Similar to colitis model, GRK6 deficient mice also suffer from increased weight loss and severity in the arthritis model.

Together, these studies demonstrate critical roles of GRKs in neurodegenerative as well as autoimmune disease processes and thus provide rationale for targeting these GRKs and their associated pathways for therapeutic development.

Role of GRKs in Cardiovascular diseases

GRK2, GRK3 and GRK5 are well-known for their role in cardiovascular disease [129, 130]. Catecholamine signaling *via* β-adrenergic receptor signaling predominates during heart failure in order to improve myocardial contractility and cardiac output. However, increased expression/activity of GRK2 and GRK5 during heart failure induces β-adrenergic receptor desensitization. This reduces myocardial contractility and cardiac output and therefore worsens heart failure[131]. This further triggers a surge of catecholamines leading to a vicious

cycle of persistent β-adrenergic receptor desensitization. Overexpression of GRK2 and GRK5 *in vivo* has been shown to decrease myocardial contractility and cardiac output in response to adrenergic stimulation suggesting an impaired adrenergic receptor signaling[131, 132]. Conversely, when GRK2, 3 and 5 were inhibited, an increase in cardiac contractility and enhanced survival were observed in heart failure models[129, 133]. In addition, competitive inhibition of GRK2 or GRK5 using βARKct, (C-terminal peptide of GRK2) or adGRK5-NT (N-terminal peptide of GRK5) respectively, prevented cardiomyopathy and improved heart failure[134-137]. Furthermore, overexpression of GRK2 and GRK5 in vascular smooth muscle cells (VSMCs) led to development of hypertension[138, 139]. In addition to these GPCR-dependent roles, GRK5 by virtue of its nuclear localization signal has also been shown to accumulate in the nucleus of cardiomyocytes and function as a histone deacetylase (HDAC) kinase and promote cardiac hypertrophy[25]. GRK2 and -5 have also been implicated in the development of atherosclerosis. LDL receptor knockout mice with partial GRK2 deficiency in hematopoietic cells develop fewer atherosclerotic plaques[90]. Partial GRK2 deficiency leads to increased mobilization of macrophages to inflammatory sites leading to plaques with smaller necrotic core. In contrast to GRK2, GRK5 deficiency in ApoE knockout mice develop more aortic atherosclerosis[98].

Overall, studies in the past decade clearly demonstrate GRK inhibition has direct beneficial effects in cardiovascular disease conditions by promoting survival signals. Thus several research groups have embarked on identifying GRK2 and GRK5 inhibitors for therapeutics in heart failure.

Sepsis

Sepsis and systemic inflammatory response syndrome (SIRS) are leading causes of mortality in intensive care unit[140]. GPCRs play a major role in the pathophysiological events in sepsis, by regulating cardiovascular, immune and coagulatory responses. GRK2 and GRK5 play a significant role in the pathogenesis of human sepsis[55, 92, 141-143] by regulating neutrophil chemotaxis. In addition, mouse models of polymicrobial sepsis[92, 143, 144] and endotoxemia[53, 142] implicate roles for GRK2 as well as GRK5 in regulating outcomes of septic shock. Studies from our lab demonstrated that myeloid specific deficiency of GRK2 leads to enhanced cytokine production in endotoxic shock model. This was shown to be via negative regulation of NF-κB1p105-TPL2-MEK-ERK pathway by GRK2[53]. Consistent with these results, myeloid specific GRK2 knock mice also exhibit exaggerated cytokine response in polymicrobial sepsis model[143]. However, GRK2 deficiency in myeloid cells didn't affect immune cell infiltration or bacterial presence. Additionally, enhanced cytokine levels in GRK2 knockout mice did not significantly affect survival.

Compared to GRK2, our studies demonstrated that GRK5 deficiency leads to diminished cytokine levels in both endotoxemia and polymicrobial sepsis models[46, 142, 144]. As discussed previously GRK5 deficiency led to decreased NFκB activation in tissues as well as in macrophages. In addition to reduced cytokine responses, GRK5 deficient mice also showed decreased thymocyte apoptosis and immune suppression[144]. Furthermore, decreased immune suppression was attributed to reduced plasma corticosterone levels in GRK5

deficient mice. Overall, these effects protected GRK5 deficient mice from sepsis-induced mortality especially in the presence of antibiotics. Role of other GRKs in sepsis is not well known and is an area of investigation in our laboratory. Given these effects of GRKs in various cellular processes that impact sepsis pathogenesis, GRKs are also potential therapeutic targets in sepsis.

Conclusion

GRKs play numerous physiological roles to maintain homeostasis. Derangement in the processes involving GRKs often leads to pathology. Pathophysiological role of GRKs are attributed both to their canonical GPCR-dependent functions as well as to their non-canonical functions. As more and more novel interactions of GRKs with non-GPCR substrates are uncovered, GRKs are becoming diverse targets for many disease conditions. Thus it is critical to take into consideration GRKs' substrate/interaction diversity, as these kinases are targeted for therapeutic purposes.

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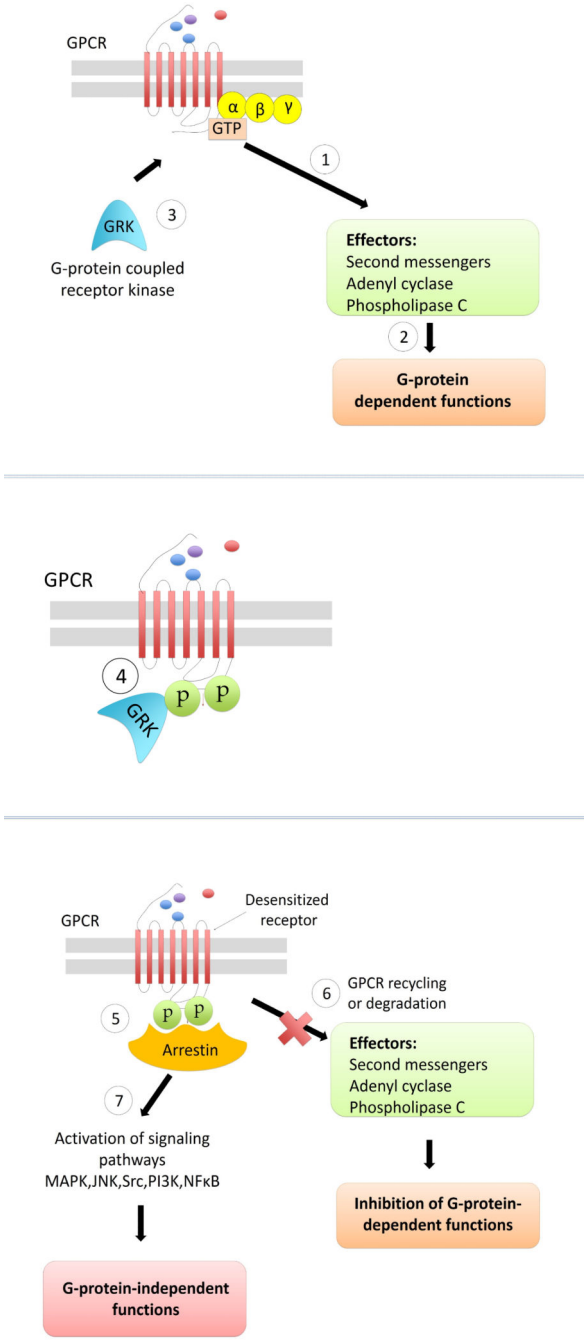

Figure 1. Schematic summary of the role of GRKs in G-protein-dependent and independent functions

a) GPCR activation and G-protein dependent function. b) GRK recruitment and phosphorylation of GPCR. c) Binding of arrestin, formation of signalosome complexes leading to GPCR desensitization and G-protein-independent functions.

1. GPCR stimulation leads to activation of various second messengers.
2. Second messengers activate downstream signaling, referred to as G-protein-dependent functions.

3. Stimulation of GPCR and its signaling activates GRKs, which are recruited to GPCRs.
4. GRKs phosphorylate intracellular domains of GPCRs.
5. Arrestin binds to phosphorylated GPCRs.
6. Arrestin binding stops GPCR-G protein signaling and leads to, in many cases, arrestin-GPCR signalosome complex. This not only inhibits G-protein-dependent signaling but paves way for G-protein-independent functions (7).

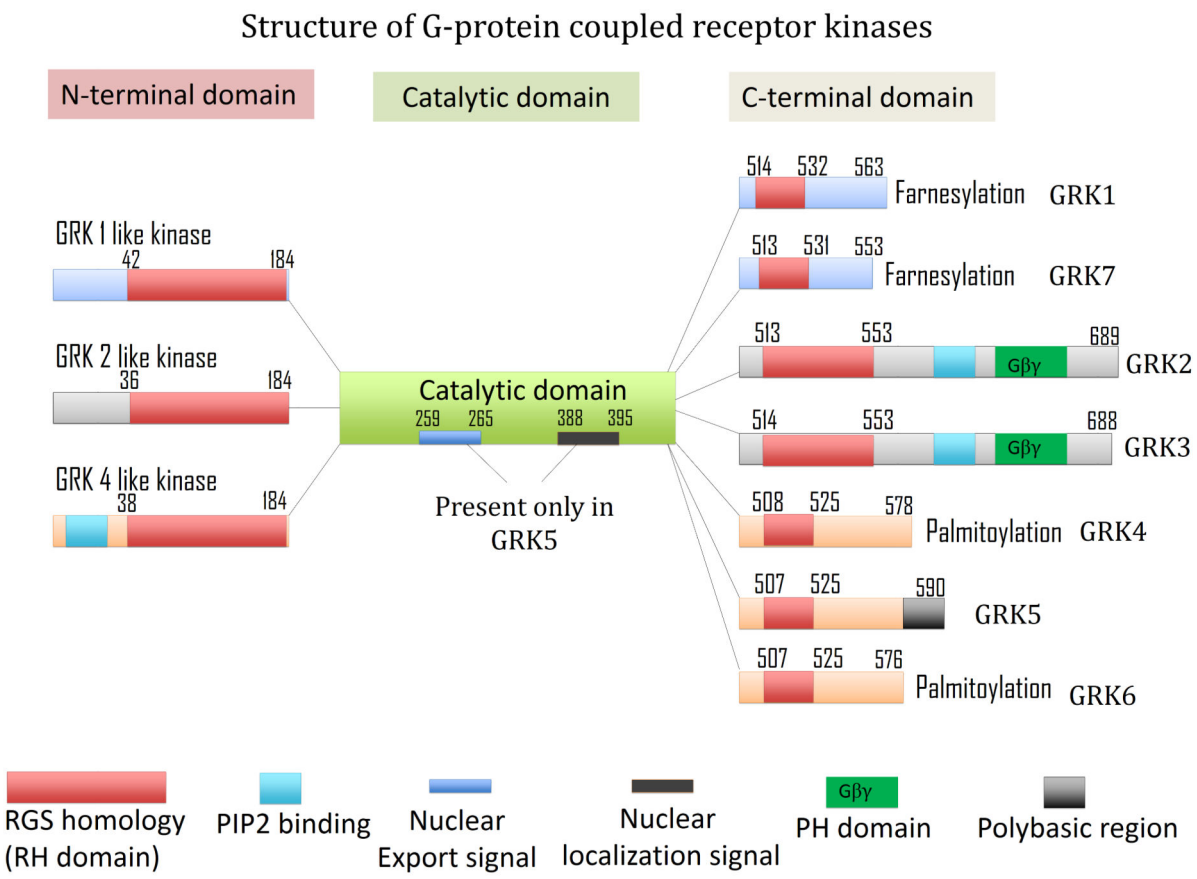

Figure 2. Domain structure of GRK family of proteins

GRK have a short N-terminal domain, a catalytic domain, and a variable C-terminal domains. See text for further information. Numbers above domains represent amino acid residue based on Lodowski et al. (2006)[13]

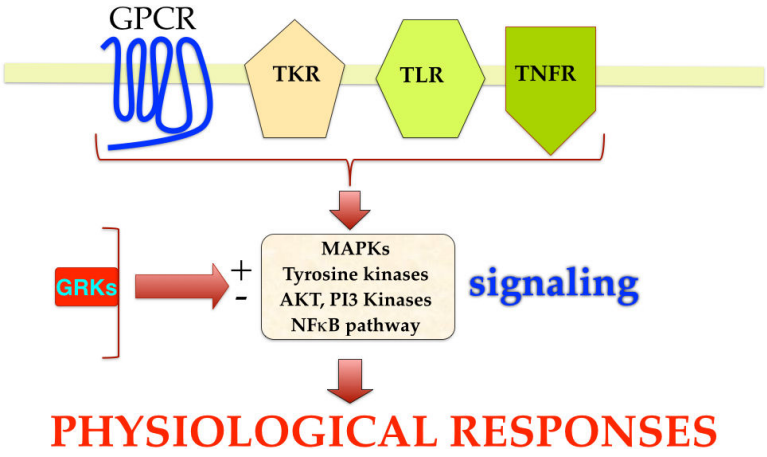

Figure 3. Emerging role of GRKs in GPCR and non-GPCR signaling pathways

Similar to arrestins, GRKs are now known to regulate multiple signaling pathways stimulated by multiple receptor classes. Whether GRKs mediate or inhibit these signaling processes depend on the receptor that is activated as well as the cell type.

Mouse phenotypes

GRKs	Phenotype	Phenotype	References
GRK2	Whole body knock out	Embryonic lethal because of its role in cardiac development	(Jaber, Koch et al. 1996)
	Heterozygote GRK2	Increase in lymphocyte chemotaxis toward the CCR5 ligand CCL4. Early onset experimental auto immune encephalomyelitis with increased infiltration of the CNS by lymphocytes and macrophages	(Vroon, Heijnen et al. 2004) (Vroon, Kavelaars et al. 2005)
	Myeloid specific knock out	Increased inflammation during endotoxaemia and polymicrobial sepsis Decreased atherosclerotic lesions in LDL-myeloid GRK2 dual knock out mice	(Parvataneni, Gonipeta et al. 2011; Patial, Saini et al. 2011) (Otten, de Jager et al. 2013)
	Vascular smooth muscle specific overexpression	Attenuated βAR-mediated signaling in VSM cells and in vivo vasodilation, increased resting blood accompanied by vascular thickening and cardiac enlargement	(Eckhart, Ozaki et al. 2002)
	Cardiac specific knock out	Enhanced inotropic sensitivity to isoproterenol, enhanced cardiac contractile performance, and reduced ventricular remodeling post infarction	(Matkovich, Diwan et al. 2006; Raake, Vinge et al. 2008)
	Adrenal specific knock out	Attenuates heart failure progression and improves cardiac function post myocardial infarction	(Lymperopoulos, Rengo et al. 2010)
GRK3	Whole body knock out	Loss of olfactory receptor desensitization and neuropathic pain induced opioid tolerance	(Terman, Jin et al. 2004) (Peppel, Boekhoff et al. 1997)
GRK4	Complete knock out	Normal fertility and sperm function. No obvious phenotype	(Virlon, Firsov et al. 1998)
GRK5	Whole body knock out	Enhanced hypothermia, hypoactivity, tremor and salivation by oxotremorine	(Gainetdinov, Bohn et al. 1999)
		Decreased NFKB activation in thioglycollate induced peritoneal macrophages and cardiomyocytes	(Patial, Shahi et al. 2011; Islam, Bae et al. 2013; Packiriswamy, Lee et al. 2013)
		Increased NFKB activation in endothelial cells	(Sorriento, Ciccarelli et al. 2008)
		Increased apoptotic response to genotoxic damage Decreased thymocyte apoptosis during sepsis	(Chen, Zhu et al. 2010) (Packiriswamy, Lee et al. 2013)
	Myocardial overexpression	Attenuation of contractility and heart rate in response to β agonist	(Rockman, Choi et al. 1996)
	Vascular smooth muscle specific overexpression	VSM-specific overexpression of GRK5 increases blood pressure by regulating β1AR and Ang II receptors	(Keys, Zhou et al. 2005)
GRK6	Whole body knock out	Enhanced locomotor stimulating effects of cocaine and Amphetamine Impaired T lymphocyte chemotaxis Enhanced neutrophil chemotaxis	(Gainetdinov, Bohn et al. 2003) (Eijkelkamp, Heijnen et al. 2007) (Vroon, Heijnen et al. 2004)