G-protein Coupled Receptor Kinase 2 and Murine Circadian Clock Regulation

by

Neel Mehta

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Department of Cell and Systems Biology
University of Toronto

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Abstract

G-protein coupled receptor kinase 2 (GRK2) is a critical component in the regulation of numerous intracellular signaling pathways. However, its contribution in mammalian circadian clock functioning has not been investigated. Here, the absence of GRK2 in mice was found to lengthen the circadian period of locomotor activity rhythms and delay the process of re-entrainment following jetlag, while augmenting the phase delaying response to a light-pulse during the early night. GRK2 was found to negatively regulate *mper1* transcriptional activation and nuclear localization. *In vitro* studies showed a Casein Kinase 1 (CK1)-dependent regulatory role of GRK2 on PERIOD1 localization. GRK2 activity was found to be necessary for VIP-induced internalization of VPAC2 receptor. In the absence of GRK2, the expression of VPAC2 receptors within the SCN was found to be elevated. These findings identify GRK2 as a regulator of circadian clock speed and photic entrainment via a potential VPAC2-cAMP/PKA-CK1 signaling pathway.
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1 Introduction

1.1 Circadian Rhythms

The environmental variables present on Earth pose a serious challenge on the survival of most living organisms. Such challenges have led to the evolution of intrinsic timekeeping mechanisms that help organisms cope with predictable cyclic changes within the external environment. In mammals, these timekeeping mechanisms drive circadian rhythms in biochemical, metabolic, physiological and behavioral processes such as sleep-wake cycles, cell cycle progression, thermoregulation, glucose metabolism and locomotor activity rhythms. Being central to the regulation and optimal functioning of a vast array of diverse biological processes, circadian dysregulation has significant impact on human health and has been shown to be correlated with a myriad of diseases such as cancer, metabolic syndrome and cardiovascular dysfunction.

Circadian rhythms are processes that are defined as being endogenously generated with a period of approximately 24 hours. Being endogenously generated, a circadian rhythm must persist even in the absence of any external timing cues (zeitgebers) such as the light-dark (LD) cycle. Such endogenously driven circadian rhythms are defined as free running. In addition to its autonomous nature, a circadian rhythm must be able to acutely reset in response to external timing cues such as light. This process known as entrainment allows an organism to readily anticipate, synchronize and adapt to the daily recurring changes in the external environment.

The timekeeping mechanism responsible for generating circadian rhythms consists of three components (Fig 1):

1. **Input Pathways** that receive and transmit environmental cues such as light to a central oscillator.

2. **Central Oscillator** that generates and maintains circadian rhythmicity.

3. **Output Pathways** consisting of downstream clock regulated effectors that are manifested at the organismal level.
1.2 The Central Oscillator - The Suprachiasmatic Nucleus: Mammalian Master Pacemaker

In mammals, the hypothalamic suprachiasmatic nucleus (SCN) serves as the central oscillator that autonomously generates and maintains organismal-level rhythmicity with approximate 24-hour periodicity\(^2\). The SCN is a bilateral hypothalamic structure located above the optic chiasm and anatomically separated by the third ventricle\(^2\). Defined based on the inputs and outputs, the SCN can be subdivided into shell and core compartments\(^6,7\). The core SCN receives retinal input from the retinohypothalamic tract and possesses vasoactive intestinal polypeptide (VIP) and/or gastrin releasing peptide (GRP) expressing neurons\(^6\). The shell SCN is comprised of arginine vasopressin (AVP) expressing neurons that are essential for generating circadian rhythmicity\(^6,7\). To control circadian outputs, the SCN transmits signals to peripheral clocks within the brain via neuronal and humoral mechanisms that in turn coordinate timing within peripheral tissue oscillators located outside of the brain, for example in the liver and kidneys\(^7\).

The bilateral SCN is composed of approximately 20,000 neurons\(^8\). Within these neurons reside the single-cell oscillators that generate their own endogenous circadian rhythmicity\(^3,5\). At the molecular level, the individual circadian oscillators are tightly coupled to each other and are modeled as a simplistic, interlocked, auto-regulatory transcription/translation feedback loop consisting of a set of core-clock genes\(^3,5\).

1.3 The Core Molecular Clockwork

The molecular clockwork, which consists of auto-regulatory transcription/translation feedback loops, drives circadian rhythms of expression within a subset of the essential core clock genes (Fig 1)\(^3,9,10\). In mammals, the basic helix-loop-helix transcription factors, CLOCK and BMAL1 create the positive limb of the feedback loop by forming heterodimers within the nucleus that activate the E-box mediated transcription of the transcriptional repressors period (per) and cryptochrome (cry) genes (Fig 1)\(^1,3,11\). PER and CRY act as the negative limb of the feedback loop, accumulating within the cytoplasm and consequently translocating to the nucleus to repress their own E-box regulated CLOCK/BMAL-mediated transcription\(^1,3,11\). Following degradation of PER/CRY proteins, CLOCK/BMAL1 function is allowed to resume (Fig 1)\(^1,3,11\). Of note, BMAL1 expression is regulated by a secondary feedback loop involving the CLOCK/BMAL1-activated transcription of retinoic-acid-related orphan nuclear receptors Rev-erba and Rora (Fig
1) Via competitive binding of retinoic-acid-related orphan receptor response elements (ROREs) present within the \textit{bmal1} promoter, REV-ERB\textalpha{} and ROR\textalpha{} proteins either repress or activate \textit{bmal1} transcription, respectively (Fig 1)\textsuperscript{12,13}.

These cellular oscillators ultimately drive circadian rhythmicity at the organismal level by regulating the expression of downstream \textit{clock-controlled genes} such as arginine vasopressin\textsuperscript{1}. Although the transcription-translation feedback loop is primarily responsible for generating circadian rhythmicity, posttranscriptional and posttranslational mechanisms are considered to be critical in modulating and fine-tuning clock function. For example, the post-transcriptional regulatory role of miRNAs in circadian clock functioning is becoming increasingly understood and appreciated\textsuperscript{14}. Other than miRNA regulation, post-translational modifications such as phosphorylation of clock proteins also play an important role in regulating their turnover rate, stability and subcellular localization\textsuperscript{15,16}. These events ultimately set the speed of the circadian clock\textsuperscript{16}. For example, casein kinase 1 delta (ck1\textdelta{}) is a kinase responsible for regulating the 24 hour period and pace of the circadian clock by phosphorylating PER and delaying its accumulation and nuclear entry and consequently increasing its turnover via the ubiquitin-proteasome pathway\textsuperscript{16,17}. Here, the importance of these kinases in clock function has been illustrated by the use of CK1 inhibitors. Blocking the function of CK1\textdelta{} and CK1\textepsilon{} using PF-670462 has been shown to significantly diminish PER phosphorylation, promote nuclear retention, inhibit PER degradation, and result in period lengthening via possibly prolonging the PER2 transcriptional feedback loop \textsuperscript{18-20}. Such post-translational events involving PER accumulation and degradation result in a lag between the positive and negative limbs of the core transcription/translation feedback loop\textsuperscript{16}. This lag between the two elements is primarily responsible for producing rhythmic clock gene expression with approximate 24 hour rhythmicity\textsuperscript{16}. Interestingly, over the recent years, mathematical and computational models have shown that phosphorylation of the protein, PER, has site-specific effects at the clock level\textsuperscript{18}. For instance, phosphorylation of different sites on PER by casein kinase 1 (CK1) and glycogen synthase kinase 3 can lead to opposite effects on PER stability and on the circadian period\textsuperscript{18}. More specifically, phosphorylation at one site enhances the rate of degradation of PER and decreases the circadian period, while phosphorylation at another site stabilizes PER, enhances the transcription of \textit{per}, and increases the circadian period\textsuperscript{18}. The roles of GSK3 in circadian clock regulation are also becoming increasingly appreciated. Blocking GSK3 using lithium salt
has been known to result in period lengthening and an increase in clock gene transcription, allowing for increased amplitude and synchrony of clock gene oscillations\textsuperscript{21}. To further illustrate the importance of rhythmic clock gene expression, mutations of clock gene often result in clock arrhythmicity or the production of an abnormal free-running circadian period at the behavioral level\textsuperscript{22-24}. For example, mice lacking \textit{per1} or \textit{per2} exhibit shorter free-running periods at the level of locomotor activity, with some \textit{per2}-deficient mice eventually becoming arrhythmic\textsuperscript{22-24}. On the other hand, overexpression of \textit{per1} has been shown to result in an increase in period length\textsuperscript{25}.
Figure 1. Mammalian Circadian System.

A) Diagram showing the basic components of a mammalian circadian system. The three components making a circadian system consist of the central pacemaker that drives endogenous oscillations, the input pathways that relay environmental timing cues to the pacemaker to adjust its phase or period and the output pathways regulated by the pacemaker that control overt circadian rhythms in physiological and behavioral processes. In mammals the retinohypothalamic tract is the major input pathway originating from photoreceptive cells within the retina that conveys environment information such as light to the suprachiasmatic nucleus (SCN). As the central pacemaker, the SCN coordinates outputs of physiological and behavioral circadian rhythms in peripheral oscillators throughout the body.

B) In mammals, the molecular mechanism responsible for generating circadian rhythmicity consists of a transcriptional/translational auto-regulatory feedback loop. The transcription factors BMAL1 and CLOCK form heterodimers that activate the expression of Period (per) and Cryptochrome (cry) along with Rev-erba and Rora. CRY and PER constitute the negative limb of the primary feedback loop: following their heterodimerization in the cytoplasm, PER/CRY translocate into the nucleus where they repress their own BMAL/CLOCK-mediated transcription. Rhythmic bmal1 expression is also regulated by an ancillary transcriptional/translational feedback loop consisting of REV-ERBα and RORα proteins that can either repress or activate bmal transcription, respectively. Phosphorylation of PER/CRY proteins play an important role in regulating their turnover rate, stability and subcellular localization and in maintaining 24 hour circadian rhythmicity within the molecular feedback loop.
1.4 Photic Entrainment

Even though circadian rhythms are persistent in constant environmental conditions, environmental cues such as the light-dark (LD) cycle and discrete light pulses are known to entrain or reset the SCN clock. Entrainment is a process by which the phase of the internal circadian clock is synchronized to an environmental oscillation such as the LD cycle. Such an ability for the clock to run autonomously while being able to be reset by external cues greatly enhances the fitness of organisms by improving their ability to predict and adapt to daily recurring events in the external environment.

Photic entrainment requires daily phase adjustments in the clock that is dependent on the phase response curve (PRC) of the particular clock being examined for light at a given intensity. A PRC is an intrinsic, species-specific, oscillator-specific tool used to depict the sensitivity, extent and direction to which an entraining stimulus such as light is capable of resetting a circadian rhythm. For light of a given intensity and duration, the PRC describes the direction and magnitude of the phase shift (i.e. phase advance or delay) that will take place based on the circadian phase at which the light stimulus is administered. For example, a short 15 minute light pulse given during the early subjective night or late subjective night for a free-running (housed in constant darkness) nocturnal mouse will rapidly undergo entrainment by phase delaying or phase advancing the clock, respectively, at the clock gene expression and behavioral locomotor activity levels. However, light administered during the daytime will fail to elicit a phase shift.

1.5 Molecular Basis of Photic Entrainment

Light is the most potent zeitgeber capable of entraining the SCN clock. Photic information from the environment is relayed from the retina to the SCN via the monosynaptic retinohypothalamic tract (RHT). Light triggers the release of the neurotransmitters glutamate and pituitary adenylate cyclase activating peptide (PACAP) from the RHT nerve terminals.

Glutamate and PACAP act at the postsynaptic terminals located within the core SCN to trigger the activation of a number of protein kinase pathways such as the p42/p44 mitogen-activated protein kinase/extracellular signaling kinase (MAPK/ERK) signaling pathway.
Mechanistically, the activation of a small GTPase, Ras, results in the activation of a MAPKKK, Raf1. Raf1 phosphorylates the MAPKK, MEK1/2. MEK1/2 in turn phosphorylates and activates the terminal kinase, ERK1/2. Within the SCN, phosphorylated ERK (pERK) phosphorylates various cytoplasmic and nuclear target substrates, resulting in events such as Ca\(^{2+}\)/cAMP response element binding protein (CREB) - CRE-mediated transcriptional activation and activation of ERK-regulated kinases including p90 ribosomal S6 kinase [RSK] and mitogen and stress dependent kinase [MSK].

Evidence has shown that light induced MAPK/ERK activation within the SCN is accompanied by the activation of immediate early genes such as c-fos and activation of cAMP response element binding protein (CREB) dependent transcription of clock genes such as per1 and per2. Induction of per1 and per2 is believed to underlie light-evoked clock resetting at the behavioral level, with data suggesting a specific contribution of per1 and per2 to phase advances and phase delays, respectively. However, it is important to realize that light induced c-fos activation, clock gene activation and behavioral resetting are not always an inter-dependent phenomenon. Furthermore, a light stimulus can have differing effects at the molecular signaling level depending on the time of the night it is administered. During the early night, a light pulse activates calcium/nitric oxide mediated signaling, resulting in MAPK/ERK signaling activation, CREB/CRE mediated transcriptional activation and a PER2 dependent phase delay at the behavioral level. During the late night, a light pulse activates a cGMP/Protein Kinase G dependent, ryanodine receptor independent signaling, resulting in MAPK/ERK signaling activation, CREB/CRE mediated transcriptional activation and a phase advance at the behavioral level. Interestingly, activation of cAMP/PKA has been found to enhance light/glutamate signaling events and clock state resetting during early night while blocking or opposing light/glutamate-induced clock resetting in the late night.

In vivo light stimulation or glutamate administration has been shown to result in large and rapid increases in ERK phosphorylation within the murine SCN. ERK activation is then followed by the activation of downstream components such as c-fos, CREB and per1 and per2. Light pulses given during dead-zones such as the subjective day are blocked (gated) and fail to activate the ERK signaling cascade, and as a result fail to alter the clock phase at the gene and behavioral level.
Conversely, blocking the MAPK/ERK pathway using specific inhibitors within the SCN has been shown to completely abolish light induced ERK activation and all downstream outcomes, including CREB activation, clock gene activation and phase shifting at the behavioral level\textsuperscript{32}.

The importance of MAPK/ERK signaling activation as a marker for light-induced clock resetting is further strengthened by research suggesting that ERK activation itself is under tight regulation within the SCN\textsuperscript{29,39,41}. For example, studies have shown that PKC-mediated Raf kinase inhibitor protein (RKIP) phosphorylation regulates MAPK/ERK signaling and plays a role in mediating photic entrainment within the murine SCN\textsuperscript{29}. More specifically, it has been shown that unphosphorylated RKIP binds to and inhibits Raf1, preventing Raf1 from phosphorylating MEK and activating downstream MAPK/ERK signaling components\textsuperscript{29}. However, upon light induced-PKC phosphorylation, PKC is now able to phosphorylate RKIP\textsuperscript{29}. RKIP phosphorylation results in the dissociation between RKIP and Raf1\textsuperscript{44}. Consequently, RKIP dimerization converts RKIP from a Raf1 inhibitor to a G-protein coupled receptor kinase-2 (GRK2) inhibitor\textsuperscript{44}.

1.6 G-Protein Coupled Receptor (GPCR) Signaling within the SCN

GPCRs are a class of receptors with seven transmembrane domains that detect external signals and transduce them within a cell to elicit a biological response\textsuperscript{45}. In the case of GPCR signaling, intracellular signaling pathways via secondary messengers and through cross-talk can mediate the transmission of extracellular signals to various effectors\textsuperscript{45}. These effectors are then capable of activating a myriad of biological responses\textsuperscript{45}. Mechanistically, upon agonist binding, the interacting trimeric G-proteins are dissociated into Go and Gβγ subunits\textsuperscript{45}. Here, these G-proteins can either activate or inhibit their own secondary downstream effectors such as adenylate cyclase\textsuperscript{45}. The SCN is known to contain a variety of G-protein coupled receptors (GPCRs) that can initiate critical intracellular signaling events that are responsible for mediating clock function and critical for the process of light-induced clock resetting.

1.6.1 Pituitary adenylate cyclase activating polypeptide Type 1 Receptor

Pituitary adenylate cyclase activating polypeptide (PACAP) is a member of the vasoactive intestinal polypeptide (VIP) family of neuropeptides localized within a subset of neurons within the RHT\textsuperscript{30}. PACAP is co-stored and co-released with glutamate from the RHT\textsuperscript{30}. Both in vitro
and in vivo studies have suggested that the phase of the circadian rhythm within the SCN can be regulated by PACAP\cite{42}. Within the SCN, PACAP has been shown to modulate glutamatergic signaling in a time- and concentration-dependent manner in order to regulate clock gene expression via activating various intracellular signaling pathways including the cAMP/PKA and PLC signaling pathways\cite{42}. Infusion of exogenous PACAP to the SCN can phase shift the clock and induce \textit{per} gene expression in the SCN, mimicking the effects of a light pulse\cite{42}.

The PACAP-preferring type 1 G-protein coupled receptor (PAC1R) is broadly expressed within the SCN\cite{42}. Mice lacking PAC1R within the SCN show abnormal phase delays and advances in response to an early and late night light pulse along with abolished early-night light-evoked activation of \textit{per1/2} and \textit{cFos} expression in the SCN\cite{42}. PAC1R knockout mice are also slower to entrain to a jetlag paradigm consisting of an 8-hour advance or an 8-hour delay in the LD cycle\cite{46}. Collectively, PAC1R signaling within the SCN has been suggested to serve as a mediator of light information and act as a gating control responsible for regulating the photic responsiveness of the clock\cite{42,46}.

1.6.2 VPAC2R

The distribution of vasoactive intestinal polypeptide (VIP) in the mammalian circadian system is different from that of PACAP\cite{47}. VIP is synthesized in a population of cells in the retinally innervated core region of the SCN\cite{47}. A large proportion of these VIP cells express the \textit{c-fos} gene and couple to CREB responsive transcriptional activation of genes such as \textit{per1} following light stimulation\cite{47}. In vivo studies have also shown that VIP acts to phase-advance the release of arginine vasopressin (AVP), a clock-controlled neuropeptide thought to play an important role in transmitting the output of the circadian clock to targets outside the SCN\cite{47}. VIP/PACAP preferring type 2 receptor (VPAC2R) is localized throughout the entire SCN, and is known to be equally sensitive to VIP and PACAP\cite{47}. VPAC2R, both at the transcript and protein level, has been shown to be rhythmic under free-running conditions, peaking during the latter part of the subjective day and in the latter part of the subjective night, respectively\cite{48,49}. Many studies have examined the roles and importance of VPAC2 and associated neuropeptidergic signaling in circadian clock functioning\cite{47}. The deficiency of VPAC2 in mice has severe consequences on circadian behavior and clock gene oscillations\cite{47}. Studies using mice lacking VPAC2 have shown that this receptor is essential for the expression of robust circadian rhythmicity\cite{47}. Mice lacking VPAC2R exhibit a complete lack of coordinated rhythmic clock gene expression in the SCN.
along with the lack of rhythmic AVP expression. Behaviorally, the lack of coordinated clock gene expression in mice deficient for VPAC2R results in weakly rhythmic locomotor activity, with no clear time of activity onset under free running conditions. On the other hand, mice overexpressing VPAC2 have been shown to exhibit a shorter free running circadian period. Overall these data suggest a critical role of VPAC2 signaling within the SCN for the regulation of photic entrainment and circadian rhythmicity.

1.6.3 Arginine Vasopressin and Arginine Vasopressin Receptor 1a and 1b

AVP-mediated interneuronal communication has been hypothesized to be critical for providing an intrinsic resistance to external perturbations like jetlag. Jetlag arises from a misalignment between the internal circadian clock and external environmental time. Recently, arginine vasopressin (AVP) and its receptors (V1a and V1b receptors) have been found to be responsible for the regulation of jet lag in mice. V1a mRNA levels exhibit a circadian expression, peaking during the subjective night within the SCN. It was found that in mice lacking vasopressin receptors V1a and V1b (V1a–/– V1b–/–) circadian rhythms at the behavioral and clock gene level re-entrained immediately or were resistant to jetlag-inducing paradigms. Interestingly, V1b knockout mice were found to be more effective in inducing a jet-lag resistance phenotype as compared to V1a knockout mice. The behavior and clock gene expression data from V1a–/– V1b–/– mice revealed a normally functioning clock mechanism that oscillated indistinguishably from WT mice under normal LD conditions. However in one instance, genetic deletion of the V1a receptor has been shown to result in an elongated free running period in mice. These studies collectively show the importance of arginine vasopressin dependent G-protein coupled signaling in interneuronal communication for providing an intrinsic barrier or resistance to clock resetting stimuli.

1.7 GPCR Signaling Regulation

Even though the importance of numerous signaling pathways consisting of GPCR associated signaling within the SCN have been acknowledged and studied extensively, it is of equal importance to examine how these signaling events are regulated.
1.7.1 GPCR signaling regulation via G-protein coupled receptor kinase 2

Once GPCR signaling is activated, the biological processes initiated by GPCR signaling must be quickly terminated. Homologous desensitization is one way by which GPCR signaling can be quickly terminated\textsuperscript{52}. Here, specific G-protein-couple receptor kinases (GRKs) can phosphorylate activated agonist-bound GPCRs and lead to a rapid desensitization of these receptors (Fig 2)\textsuperscript{52}.

GRKs constitute a family of six mammalian serine/threonine kinases that play an important role in modulating intracellular signaling. Classically, GRKs function as “off switches”, phosphorylating and desensitizing agonist-bound, activated GPCRs\textsuperscript{52,53}. All GRKs are composed of a variable extracellular N-terminal, a highly conserved central catalytic kinase domain with 3 transmembrane intracellular loops, and a poorly conserved cytoplasmic C-terminal tail region\textsuperscript{53}. The N-terminus of GRK2 contains a regulator of G protein signaling (RGS) homology domain (RH domain). RH domains are known to serve as GTPase activating proteins (GAPs) that bind to various Gαβ subunits of heterotrimeric G proteins and accelerate their GTPase activity\textsuperscript{53}. The C-terminal domain of GRK2 is composed of lipid and protein binding sites that are proposed to be important for receptor targeting\textsuperscript{53}.

GRK2 has received a lot of attention over recent years as a result of its large interactome and an increasing appreciation and understanding of the key regulatory roles it plays in a wide range of receptor mediated signaling networks involved in key cellular processes such as cell cycle progression, cell migration, insulin signaling, and cardiac function\textsuperscript{52-54}.

Internalization for most GPCRs occurs on the order of minutes and correlates with receptor phosphorylation by GRKs and subsequent β-arrestin translocation (Fig 2)\textsuperscript{52,54}. GRK2 translocation and membrane localization are mediated in part by their binding to heterotrimeric G protein βγ subunits, allowing for membrane recruitment in proximity of the activated GPCR (Fig 2)\textsuperscript{52}. Within a few seconds of continuous receptor activation, cells are able diminish the ability of an agonist to activate its GPCR and downstream G protein signaling\textsuperscript{52,54}. After continuous agonist exposure, signaling desensitization can be further augmented as a result of receptor internalization and degradation (Fig 2)\textsuperscript{52,54}. These desensitization and receptor internalization events are thought to be critical for signaling regulation and generally are known to occur with many types of GPCRs and growth factor receptors\textsuperscript{52,53,55}. Mechanistically, GRK2
binds to agonist-bound, activated GPCRs, leading to the activation of GRK2’s intrinsic kinase activity\(^5^2^\). As a result, GRK2 phosphorylates serine/threonine residues within the third intracellular loop or the C-terminal tail of the GPCR (Fig 2)\(^5^2^\). Once phosphorylated, β-arrestin proteins specifically bind the GPCR at heterotrimeric G-protein binding sites (Fig 2)\(^5^2^\). This interaction prevents the GPCR from interacting with and activating its associated G-proteins upon further stimulation with agonist (Fig 2). This steric hindrance effectively uncouples receptor activation from downstream G-protein coupled signaling activation\(^5^2^\). Furthermore, GRK2 and β-arrestin are also capable of initiating receptor-mediated endocytosis (Fig 2)\(^5^2^\). This internalization process via GRKs and β-arrestin serves as another mechanism by which GPCR signaling is dampened. However, internalized GPCRs can be dephosphorylated by phosphatases and recycled back to the plasma membrane, where the GPCR is ready to be activated again (Fig 2)\(^5^2^,^5^6^\). Here, the rates at which the receptors are recycled depend on the type of GPCR in question. Literature in this regard classifies GPCRs at either Class A or Class B, depending on the stability of the β-arrestin-receptor complex\(^5^7^\). Class A GPCRs typically form unstable complexes with β-arrestin and are rapidly recycled, whereas Class B GPCRs form stable complexes with β-arrestin within endosomes, either allowing them to be retained intracellularly for prolonged periods of time or targeting them for degradation (Fig 2). Class A receptors include arginine-vasopressin receptors V1a and V1b, while Class B receptors include VIP/PACAP VPAC2 and PAC1 receptors. Interestingly, recent evidence suggests that the GRK2, β-arrestin and GPCR signalosome can also initiate signaling pathways, such as the MAPK/ERK pathway, which exhibits a more gradual and prolonged kinetic profile, as opposed to the G-protein activated MAPK/ERK pathway activation which is more transient and relatively short-lived\(^5^4^,^5^6^-^6^0^\).

Furthermore, other than phosphorylating activated GPCRs, GRK2 is capable of interacting with several members of the receptor tyrosine kinase (RTK) superfamily, including platelet-derived growth factor receptor-β and epidermal growth factor receptor. More importantly, GRK2 is involved in both kinase-dependent and -independent interactions with a variety of non-receptor substrates such as RKIP, MEK and ERK, all of which have been shown to play a pivotal role in coupling light to the circadian clock\(^5^4^,^5^5^\). With regards to MAPK/ERK signaling, it has been shown that GRK2 is capable of both inhibiting and activating MAPK/ERK signaling in response to different agonists and at different levels of the signaling cascade\(^6^1^,^6^2^\). For example, GRK2 has
been found to interact in vitro with non-receptor substrates such as MEK and in turn inhibit chemokine-mediated MAPK/ERK activation\textsuperscript{61}. On the other hand, as previously mentioned, GRK2 can positively regulate ERK activation as a result of receptor endocytosis and consequent endosomal activation of MAPK/ERK signaling components such as Raf1\textsuperscript{53}. 
1 Desensitization

Recycling Vesicle

Rapid Recycling

Acidified Vesicle Compartment

Degradation

Desensitization

Clathrin-coated vesicle

Clathrin

Desensitization

2 Sequestration

Endosomal Vesicle

“Class A” GPCR

Endosomal Vesicle

“Class B” GPCR

Degradation

Slow Recycling?

3 Recycling / Downregulation

Leffkowitz and Lutrell 2002
Figure 2. Receptor Regulation by GRK2

Ligand/hormone binding to GPCR initiates G-protein coupled signaling. GRK2 is recruited to the agonist-bound GPCR. GRK2 phosphorylates GPCR, allowing for binding of β-arrestin at G-protein binding sites, resulting in receptor desensitization via G-protein uncoupling. Receptor-bound β-arrestin also acts as an adapter protein for recruiting proteins responsible for initiating receptor internalization. Once internalized, GPCRs exhibit two types of patterns depending on the interaction between the GPCR and β-arrestin. Class A GPCRs display weak association with and rapid dissociation from β-arrestin upon internalization; these receptors are quickly dephosphorylated and recycled back to the plasma membrane. Class B receptors form stable GPCR-β-arrestin complexes, accumulate within endocytic vesicles, and are targeted for degradation.
1.7.2 Mechanisms of GRK2 Regulation

GRK2’s role in GPCR desensitization is highly complex, variable, and subjected to tight regulation itself via various kinase and non-kinase partners. GRK2’s activity has been shown to be both positively and negatively regulated. PKC-mediated phosphorylation of RKIP converts RKIP from a Raf1 inhibitor to a GRK2 inhibitor: instead of binding to and inhibiting Raf1, phospho-RKIP binds to GRK2 and inhibits its activity\(^{44}\). However, PKC also phosphorylates GRK2 at serine (Ser) 29. Ser-29 phosphorylation positively regulates the activity of GRK2 by inhibiting the calmodulin-GRK2 association\(^{62}\). Calmodulin binds to and inhibits the kinase activity of GRK2 in its Ser-29 unphosphorylated form. \(^{62}\) Hence, PKC both indirectly regulates GRK2 via its action upon RKIP in a negative manner and directly regulates GRK2 via its phosphorylation at Ser-29 in a positive manner. Secondly, PKA is known to phosphorylate GRK2 at Ser-685\(^{63}\). Ser-685 phosphorylated GRK2 exhibits enhanced G\(\beta\gamma\) binding and receptor desensitization\(^{63}\). As a result, PKA-mediated GRK2 phosphorylation positively regulates GRK2 activity\(^{63}\). Lastly, ERK1/2 phosphorylates GRK2 at Ser-670. Ser-670 phosphorylation negatively regulates GRK2 kinase activity and as a result, diminishes GRK2’s ability to phosphorylate GPCRs, an event that could result in enhanced ERK activation and serve as a potential feedback inhibition mechanism for GRK2 activity\(^{64}\).

Unfortunately, even though the interplay between PKC, RKIP and ERK signaling has been well characterized, the contribution of GRK2 within this web of signaling events has not been examined.

1.7.3 Examples of GRK2 Mediated Signaling Events

GRK2 has been shown to be critically important for regulating vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide receptors (VPAC2) within smooth muscle cells. VIP-induced phosphorylation of GRK2 by PKA has been shown to result in increased GRK2 activity and binding to VPAC2. This increase in GRK2 activity correlates with an increase in receptor desensitization, internalization and degradation\(^{63}\). Conversely, a decrease in VIP-induced VPAC2 phosphorylation, receptor desensitization, internalization and degradation could be achieved either by blocking PKA phosphorylation of GRK2 using a PKA inhibitor or a GRK2 point mutation (S685A), or by blocking VPAC2 phosphorylation by GRK2 using a kinase deficient form of GRK2 (K220R) \(^{63}\).
In another aspect, melanopsin mediated signaling has been shown to be involved in non-image forming processes such as circadian entrainment and melatonin synthesis. Melanopsin signaling is known to be dispensable at the input level of the circadian clock. However, melanopsin based signaling is known to greatly influence the magnitude and ability of photic stimuli to entrain or reset the clock. Recently, GRK2 has been characterized as a melanopsin based phototransduction regulator. Here, GRK2 is known to phosphorylate melanopsin in a light-dependent manner. This GRK2 mediated phosphorylation event has been shown to be involved in regulating melanopsin signaling kinetics and downstream G-protein signaling.

Interestingly, even though GRKs are primarily GPCR kinases localized within the cytoplasm and plasma membrane, some GRKs have been implicated in a broad range of molecular events independent of their primary role in receptor regulation. These secondary roles can be attributed to their intrinsic kinase activity and cellular localization within regions other than the cytoplasm and the plasma membrane. For example, GRK5 is expressed within the nucleus and possesses class II histone deacetylase (HDAC) kinase activity. Research has shown that GRK5’s nuclear histone deacetylase activity serves as a transcriptional regulator of Gαq-regulated genes, and in conjunction to its primary role in β-adrenergic receptor desensitization, serves to protect against heart failure. In recent studies, GRK2 has been found to interact with and regulate the activity of HDAC6. HDAC6 serves as a α-tubulin deacetylase that is involved in cell motility and adhesion. Research has shown that GRK2 associates with and phosphorylates HDAC6 in order to stimulate HDAC6 deacetylase activity at specific subcellular locations. In one instance, stimulation of HDAC6 by GRK2 at the leading edge of migrating cells has been shown to be critical for cellular motility.

Even though GRK2 is recognized as a critical regulatory component within a myriad of cellular contexts and processes, its contribution in the regulation of the circadian clock has not been investigated to date. The aims of the experiments collectively presented within this thesis are to identify the roles of GRK2 within the murine circadian clock.
2 Materials and Methods

2.1 Animals

2.1.1 Conditional GRK2 Knockout Mice

GRK2\textsuperscript{flox/flox} mutant mice possessing \textit{loxP} sites flanking exons 3-6 of the targeted GRK2 gene (The Jackson Laboratory) were obtained and crossed with Vgat-ires-Cre transgenic mice (The Jackson Laboratory) mice to generate GABAergic neuron specific GRK2-knockout mice (GRK2\textsuperscript{flox/flox} Vgat\textsuperscript{ires-Cre/+}) (Fig 4) \textsuperscript{71,72}.

2.1.2 GRK2 Heterozygous Mice

Exons 5-8 of \textit{Adrbk1} were replaced with a PGK-neomycin resistance cassette. The insertion altered the downstream reading frame of \textit{Adrbk1}. Mutant mice were backcrossed for a minimum of 8 generations onto a C57BL/6J background resulting in GRK2 heterozygous mice (GRK2 +/-) (The Jackson Laboratory) \textsuperscript{73}. GRK2 +/- are embryonic lethal and unviable.

All animal handling and experimental procedures were approved by the Animal Welfare Committees at the University of Toronto in accordance with institutional guidelines.

2.2 Behavioral Experiments

Male mice ranging from 2-3 months of age at the start of the experiment were housed in cages equipped with running wheels placed into a ventilated activity chamber with controllable light schedules. Experimental evidence has shown that high intensity light pulses often saturate behavioral phase shift responses and thus make it difficult to assess changes in the photic sensitivity of the clock\textsuperscript{74}. Hence for all behavioral experiments, light intensities (lux) are used that have previously been tested and deemed to be sufficient in inducing the desired outcomes at the clock and behavioral level without saturating and consequently concealing any subtle phenotypes. Mice were initially entrained to a 12 h-light:12 h-dark (LD - 80 lux) cycle for at least 14 days and then transferred into a 7-hr advanced 12:12 LD cycle. Once stably entrained to the new LD cycle, mice were released in complete darkness (DD) to measure free-running circadian period. After about 10-14 days in DD, mice received a brief light pulse (30 lux) for 15 min at circadian time (CT) 15. Mice were then allowed to phase shift and free run for at least 10-14 days in DD, at which point they were placed back on a 12:12 LD (80 lux) cycle for at least
10-14 days until stably entrained. Once stably entrained to the new LD cycle, mice were released in complete darkness for about 10-14 days, at which point the mice received a brief light pulse (40 lux) for 15 min at CT 22. Mice were allowed to phase shift and free run for at least 10-14 days in DD prior to experiment termination. Mice had ad libitum access to food and water throughout all experiments.

2.2.1 Behavioral Experiments –Analysis

Behavioral analyses were performed with the ClockLab software (Actimetrics Inc). Endogenous period was measured using at least 14 days in constant darkness by drawing a regression line through daily activity onsets (n=20 wt, n=14 GRK2 +/-, n=12 GRK2Flox/Flox Vgatires-Cre/+). Light-induced phase shifts were measured by fitting regression lines through the daily activity onsets before and after the light pulse. The resulting change in displacement was measured and recorded as the phase shift (n=20 wt, n=14 GRK2 +/-, n=12 GRK2Flox/Flox Vgatires-Cre/+). Cumulative phase shifts to the 7-hr advance in the LD cycle were measured as the daily difference between the actual onset of daily locomotor activity and the projected onset based on the previous LD cycle (n=20 wt, n=14 GRK2 +/-, n=11 GRK2Flox/Flox Vgatires-Cre/+). The total number of days it took to re-entrain was recorded as the average number of days it took for mice to re-entrain to the new 7-hr advanced LD cycle (n=20 wt, n=14 GRK2 +/-, n=11 GRK2Flox/Flox Vgatires-Cre/+). The average first day shift in the 7hr-advanced LD cycle was measured by subtracting the predicted activity onset under the previous LD cycle from the actual activity onset on the first day in the new LD cycle (n=20 wt, n=14 GRK2 +/-, n=11 GRK2Flox/Flox Vgatires-Cre/+). Data were analyzed using Student–t-test or ANOVA followed by Fisher’s Least Significant Difference test (α=0.05).

2.3 SCN Tissue Protein Harvest and Western Blotting

Mice were killed by cervical dislocation. Brains were dissected and cut into 800-mm thick coronal sections containing the SCN in cooled oxygenated media using an oscillating tissue slicer. SCN was isolated from the tissue slice using a razor blade, pooled with at least 3 mice per condition per genotype (n=3), frozen immediately on dry ice and stored at -80°C until further use. Tissues were homogenized in ice-cold RIPA supplemented with protease and phosphatase inhibitors with a pestle and incubated on ice for 20 mins and centrifuged at 4°C at 17,000 x g for
20 mins. The supernatant was collected and protein concentration was determined using the Bradford assay (ThermoFisher Scientific). Protein lysates were mixed with SDS loading buffer to 1x concentration, heated at 95°C for 5 mins, and centrifuged for 3 mins at 17,000 x g. 20-30mg of lysates were resolved in a SDS-polyacrylamide gel for approximately 2h at 100V at room temperature and electroblotted onto polyvinylidene fluoride (Immobilon P; Millipore, Bedford, MA) membrane overnight at 4°C at 30V or 2h on ice at 80V. Protein transfer was confirmed using Ponceau S staining. Membranes were washed 3 times for 5 mins each in Tris Buffered Saline with 0.1% Triton X-100 (TBS-T). Membranes were blocked in 5% skim milk in TBS-T for 1h at room temperature, followed by overnight incubation at 4°C with one of the following primary antibodies in blocking solution: rabbit anti-GRK2 (1:1000; SantaCruz.), rabbit anti-actin (1:20,000; Sigma Inc.). Following primary antibody incubation, membranes were washed in TBS-T and incubated for 2h at RT membranes with goat anti-rabbit IgG(H+L) horseradish peroxidase (HRP) conjugated secondary antibody (1:300,000; ThermoFisher Scientific). Chemiluminescent signal was detected using the SuperSignal West Femto Maximum Sensitivity Substrate kit (ThermoFisher Scientific). Quantitation of western blots was performed using the “measure” function in ImageJ. The measure function yielded a “mean gray” value for each protein band, which were normalized to background “mean gray” values. Values are presented as mean relative abundance of the protein examined normalized to relative abundance of actin.

2.4 Tissue harvest and quantitative-real time PCR

Wild-type mice were kept in constant dark conditions for 2 consecutive days before any experimentation. For circadian expression analysis of GRK mRNA, three to four wild-type mice were sacrificed at 4-h intervals throughout the circadian cycle at CT 2, 6, 10, 14, 18, and 22 (n=3-4 mice/time point). Total RNA was extracted from individual SCN tissues using the Trizol Reagent according to manufacturer’s instructions. RNA was reverse transcribed into cDNA and diluted 1:10 and 1:100 for real-time PCR using the Absolute Blue qPCR SYBR Green Mix (ThermoFisher Scientific) for GRK and 18S ribosomal RNA control, respectively. Values were normalized for each time point to 18S ribosomal RNA abundance. Data were analyzed with ANOVA followed by Fisher’s Least Significant Difference test (α=0.05).
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<tr>
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2.5 Light treatment Procedures and Tissue harvest

Three to four mice per genotype per time point were kept in constant dark conditions for 2 consecutive days before any experimentation. For circadian expression analysis of clock proteins, PER1 and PER2 and VPAC2 receptor, mice were sacrificed at 4-h intervals throughout the circadian cycle at CT 2, 6, 10, 14, 18, and 22. For pERK activation light pulse experiments, mice received a 15 min light pulse at 80 lux at the indicated CT, placed back in DD for 15 minutes and sacrificed thereafter. Control mice kept in DD were sacrificed at the indicated CT without receiving a light pulse. For Venus-PER1 light pulse experiments, mice received a 15 min light pulse at 80 lux at the CT 15, placed back in DD for 4 hours and sacrificed at CT 19. Control mice kept in DD were sacrificed at CT19 without receiving a light pulse. For GRK2 expression analysis, mice were sacrificed at CT 6, CT 15 and CT 22 under constant darkness. In all cases, mice were killed by cervical dislocation and decapitated under dim red light. Eyes were covered with black electrical tape. Brains were dissected and cut into 800-mm thick coronal sections containing the SCN in cooled oxygenated media using an oscillating vibratome. The SCN slice was fixed in 4% paraformaldehyde in PBS for 6hr at room temperature and cryoprotected in 30% sucrose overnight at 4 °C. Cyroprotected SCN slices were cut into thin 30-µm sections using a microtome. Sections were stored in 30% sucrose at 4 °C until immunohistochemical or immunofluorescence experiments were conducted.

2.6 Immunohistochemistry (IHC)

For immunohistochemical staining, sections were washed 5 times for 5 mins each in Phosphate Buffered Saline with 0.1% Triton X-100 (PBS-T). Sections were treated with 0.3% H₂O₂ in PBS for 20 mins, washed 5 times for 5 mins each in PBS-T, blocked for 1 hr at room temperature in 10% goat serum/PBS-T, and incubated overnight at 4°C in one of the following primary antibodies in blocking solution: rabbit anti-PER1 (1:4000; gift from S. Reppert, University of Massachusetts Medical School, Worcester, MA), rabbit anti-PER2 (1:50,000; gift from D. Weaver, University of Massachusetts Medical School, Worcester, MA), rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204) (1:10,000; Cell Signal), rabbit anti-VPAC2 (1:20,000; Abcam
Inc). Following overnight incubation, sections were washed 5 times for 5 mins each in PBS-T, and incubated for 2 hr at room temperature with biotinylated goat anti-rabbit IgG (H + L) secondary antibody (1:300, Vector Laboratories Inc.). Vectastain Elite ABC Kit and Peroxidase DAB Substrate Kit (Vector Laboratories Inc) were used for immunodetection.

2.6.1 IHC Image acquisition and analysis

IHC images were captured using either the 10x or 20x objective of the Zeiss Axio Observer Z1 inverted microscope operating on the ZEN 2010 software (Zeiss, Oberkochen, Germany). For pERK and VPAC2 quantitation, using the polygon tool in ImageJ, a circle for the core SCN or a crescent for the shell SCN was drawn and the gray-scale intensity of staining for each unilateral SCN region were measured using the ‘measure’ function. Background values were obtained by selecting and obtaining a gray-scale intensity value for non-immunoreactive regions in the lateral hypothalamus. Intensity values were subtracted from background intensity values to obtain a normalized mean gray value for pERK and VPAC2 staining for each SCN. For PER1 and PER2 cell counts, images were equally assigned a minimum gray-scale threshold. The ‘analyze particle’ function in ImageJ was used to obtain an immunoreactive cell count. Three-four animals were used for analysis quantitation purposes (n=3-4). Data were analyzed with ANOVA followed by Fisher’s Least Significant Difference test ($\alpha = 0.05$).

2.7 Immunofluorescence (IF)

For immunofluorescence staining, sections were washed 5 times for 5 mins each in Phosphate Buffered Saline with 0.1% Triton X-100 (PBS-T), blocked for 1 hr at room temperature in 10% horse serum/PBS-T, and incubated overnight at 4°C in one of the following primary antibodies in blocking solution: rabbit-anti GFP (1:1000; Eusera), rabbit-anti GRK2 (1:1000; SantaCruz). Following overnight incubation, sections were washed 5 times for 5 mins each in PBS-T, and incubated in the dark for 2 hr at room temperature with the following secondary antibody in blocking solution: donkey-anti rabbit Alexa Fluor 488 (1:1000; Invitrogen). Sections were washed 5 times for 5 mins each in PBS-T and mounted and sealed using DAKO mounted media and nail polish. Slides were stored in the dark at 4 °C.
2.7.1 IF Image acquisition and analysis

Images were captured using a Zeiss Axio Observer Z1 inverted microscope equipped with a Laser Scanning Microscope (LSM) 700 module equipped with ZEN 2010 software (Zeiss, Oberkochen, Germany). Individual fluorochrome signals were collected using the multitrack setting with appropriate barrier filters using the “smart set-up” option. Images were acquired from a central focal plane of 2.3µm optical thickness using the 10x or 20x objectives. Identical settings for gain, pinhole size, and brightness were used to acquire all images of the same magnification within each experiment. For quantitation of immunoreactivity, the bilateral SCN from the micrographs were outlined using the polygon tool in ImageJ. The “measure” function yielded a “mean gray” value for immunoreactive staining within the specified region of interest. The mean gray values were normalized to background “mean gray” values obtained from surrounding non-immunoreactive regions. Three-four animals were used for analysis quantitation purposes (n=3-4). Data were analyzed with ANOVA followed by Fisher’s Least Significant Difference test (α=0.05).

2.8 In vitro cell culture studies

Neuro2A cells (ATCC) were grown on 24-well plates for immunocytochemical (ICC) experiments in DMEM (Invitrogen Canada Inc.) containing 5% fetal bovine serum (WISENT Inc.) and penicillin-streptomycin (Invitrogen Canada Inc.) at 37°C/5% CO₂ until they reached approximately 70-80% confluence. Cells were then transfected with the appropriate constructs using Lipofectamine 2000 (Invitrogen Canada Inc.). Approximately 24-48 hours post transfection, cells were treated with the specified drugs in growth media. Following drug treatments for the specified time periods, cells were harvested for downstream application and analysis.

2.8.1 Clock Protein Localization Experiments

Neuro2A cells were transfected in 24-well plates as described above with either 600ng of pcDNA 3.1 Hygro or bovine WT GRK2 (gift from Dr. Petronila Penela and Dr. Cornelius Krasel), in conjunction with either 200ng of PER1-v5, PER2-v5, CRY1-v5, or CRY2-v5 (gift from Dr. Nicolas Cermakian). 18-24 hours post transfection, ICC was conducted (section 2.8.4).
2.8.2 Roles of GRK2 in CK1 mediated Clock Protein Localization

Neuro2A cells were transfected in 24-well plates as described above with either 600ng of pcDNA 3.1 Hygro or bovine WT GRK2, in conjunction with 200ng of PER1-v5. 300nM PF-670462 was added to the growth media 18-24 hours post transfection for 4 hours. ICC was conducted following drug administration (section 2.8.4).

2.8.3 Receptor Assays

Neuro2A cells were transfected in 24-well plates as described above with either 600ng of pcDNA 3.1 Hygro, bovine WT GRK2, or dominant-negative kinase-dead GRK2 (gift from Dr. Petronila Penela and Dr. Cornelius Krasel), in conjunction with either 200ng of VPAC2-GFP (Origene) or V1bR-YFP (gift from Dr. Greti Aguilera). 18-24 hours post-transfection, VPAC2 and V1b transfected cells were treated for 20 minutes at 37°C with VIP (1 µM) and AVP (100nM) in growth media, respectively, in order to induce receptor activation and consequent internalization. Basal receptor states were examined without any ligand addition. Following all experimental treatments, ICC was conducted (section 2.8.4) to compare receptor localization for each condition between pcDNA control cells, GRK2 WT overexpressing and GRK2 K220R kinase-dead overexpressing mutant cells. Experiments were repeated 2-3 times to confirm validity.

2.8.4 ICC

Transfected Neuro2A cells (ATCC) plated on round coverslips in 24-well plates were washed in 1X PBS, and incubated in 4% paraformaldehyde in Phosphate Buffered Saline (PBS) for 15 mins at room temperature. Coverslips were washed in ice-cold 1X PBS 3 times for 5 mins each, permeabilized in PBS-T, blocked for 1 hr at room temperature in 10% horse serum/PBS-T, and incubated overnight at 4°C in following primary antibodies in blocking solution: mouse anti-v5-his (1:2000; Abcam Inc.) and rabbit anti-GRK2 (1:2000; SantaCruz) for clock protein localization experiments and rabbit anti-GRK2 (1:2000; SantaCruz) and goat-anti-GFP (1:1000; Eusera) for receptor assays. Following overnight incubation, sections were washed 3 times for 5 mins each in PBS-T, and incubated in the dark for 2 hr at room temperature with the appropriate Alexa Fluor conjugated secondary antibodies in blocking solution. Coverslips were washed 3 x 5 mins in PBS-T, incubated with the nuclear DAPI stain for 5 mins, and briefly washed in PBS and
mounted and sealed using DAKO mounted media and nail polish. Slides were stored in the dark at 4 °C.

2.8.5 ICC image acquisition and analysis

Images were captured using a Zeiss Axio Observer Z1 inverted microscope equipped with a Laser Scanning Microscope (LSM) 700 module operated with ZEN 2010 software (Zeiss, Oberkochen, Germany). Individual fluorochrome signals were collected using the multitrack setting with appropriate barrier filters using the “smart set-up” option. Images were acquired from a central focal plane using the 63x objective. Identical settings for gain, pinhole size, and brightness were used to acquire all images of the same magnification within each experiment. For quantitation purposes, using ImageJ, the polygon tool was used to outline the desired transfected cell region, and cells were counted based on whether they exhibited 1) primarily nuclear or 2) nuclear and cytoplasmic protein expression. The “measure” function was also simultaneously used to yield a “mean gray” value for immunoreactive staining intensity within the total cell and nuclear regions of interest. The mean gray values from the appropriate regions were normalized to background “mean gray” values obtained from surrounding non-immunoreactive areas and average values were taken from all experiments. At least 20-40 cells from 3 replicate experiments were utilized for analysis of cell counts (n=20-40 cells - 3 replicates) and 40-150 cells from 3 replicate experiments were utilized for analysis of fluorescence intensity (n=40-150 cells - 3 replicates). Data were analyzed with two-tailed Student’s t tests (α=0.05).
3 Results

3.1 GRK2 Expression within the SCN

By utilizing quantitative-real time PCR and immunofluorescence, the presence of GRK2 along with GRK3, 4, 5 within the murine SCN was confirmed. Within the SCN, GRK2 and GRK5 transcripts show relatively steady expression levels throughout a 24-hour circadian period, whereas GRK3 and GRK4 mRNA expression peaks around the subjective night at CT18 (Fig 3). Furthermore, in line with steady GRK2 transcript levels, using immunofluorescence, ubiquitous GRK2 protein expression was found throughout the entire SCN during the daytime, early night and late night, without any qualitative differences visible across the three time points (Fig3).
Figure 3. GRK2 mRNA and Protein Expression within the murine SCN:

A) Graph illustrating *GRK* mRNA levels across a 24 hr circadian cycle within the SCN using quantitative real time PCR. RNA was extracted from SCN samples collected from wild-type (WT) mice across the 24 hr circadian cycle. Critical threshold values across the 24 hr cycle for each sample were normalized against 18s rRNA. (n=3/time point in duplicate, error bars represent standard error mean; *p*≤0.05 vs CT 2).

B) Representative micrographs of the bilateral SCN taken from WT mice at CT2, CT6 and CT22 exhibiting ubiquitous GRK2 immunoreactivity (green), with no visible changes in protein expression across the circadian time points. White box used to show magnified region below, showing cytoplasmic- and plasma membrane-localized GRK2 expression.
3.2 GRK2 Mutant Mice

Next, in order to examine the involvement of GRK2 within the SCN clock, two strains of GRK2 mutant mice were utilized. First, GRK2 levels were eliminated using a conditional Cre/loxP knockout system. GRK2 floxed mice (GRK2 \text{ flox/flox} ) were crossed to VGAT-ires-CRE mice expressing Cre recombinase under control of a vesicular GABA transporter (VGAT) promoter (VGAT\text{-ires-Cre+/-})^{71,72}. The resulting progeny (GRK2 \text{ flox/flox} VGAT\text{-ires-Cre+/-} - referred to as GRK2 KO) exhibit >95% GRK2 knockout specifically within inhibitory GABAergic cell bodies, which make up a majority of the SCN (Fig 4). Secondly, as conventional GRK2 homozygous knockout mice are embryonic lethal, a conventional GRK2 heterozygous (referred to as GRK2 het) mice strain was utilized in order to obtain a whole-animal GRK2 mutant exhibiting >50% knockdown (Fig 4)\textsuperscript{73}. Relative expression values were obtained by normalizing immunoreactive bands to actin loading control and to GRK2 levels in WT mice.
Figure 4. GRK2 mutant mice

A) Simplified diagram representing the generation of GRK2\textsubscript{Flox/Flox} VGAT\textsuperscript{ires-Cre/+} mice by crossing GRK2\textsubscript{Flox/Flox} mice with VGAT\textsuperscript{ires-Cre/+} mice, resulting in the excision of exons 3 to 6 from the gene.

B) Immunoblot examining relative GRK2 expression in the SCN of WT, VGAT\textsuperscript{ires-Cre/+}, GRK2\textsubscript{Flox/Flox}, GRK2\textsubscript{Flox/Flox} VGAT\textsuperscript{ires-Cre/+} (GRK2 knockout) and GRK2\textsuperscript{+/+} (GRK2 heterozygous) mice (values are relative to GRK2 expression in WT mice normalized to actin; n=3 mice/genotype).
3.3 GRK2 and Regulation of Behavioral Locomotor Activity Rhythms

3.3.1 Photic Entrainment: 7-hr Advanced LD Cycle Protocol: GRK2 Mutant Mice are slower to re-entrain to a 7-hr Advanced LD cycle Jetlag Paradigm

In order to examine the roles of GRK2 in the process of photic entrainment to large changes in the LD cycle, WT and GRK2 mutant mice (refers to both GRK2 KO and GRK2 het) mice were first placed in an LD cycle consisting of the light being turned on at 8:00AM and turned off at 8:00PM (LD1). Following entrainment of the mice to LD1, the LD cycle was changed such that the light turned on at 1:00AM and turned off at 1:00PM (LD2). LD2 cycle, being a 7hr advanced LD cycle, resulted in the mice advancing their locomotor activity rhythms in the direction of the new LD cycle (Fig 5). It was found that GRK2 mutant mice were significantly slower and underwent smaller phase shifts on a day-to-day basis to entrain to the new 7 hr advanced L:D cycle as compared to WT mice: as a result, GRK2 mutant mice took significantly longer to reset their circadian clocks to match the new LD cycle (Fig 5). Furthermore, it was found that most GRK2 mutant mice exhibited a significant phase delay instead of an expected phase advance on the first day in the new 7 hr advanced LD cycle (Fig 5).
Figure 5. Photic Entrainment to a 7hr LD advanced paradigm

A) Representative double plotted actograms of WT and GRK2 mutant (KO and HET) mice stably entrained to an initial 8am ON – 8pm OFF LD cycle for at least 10 days. Following entrainment, the LD cycle was advanced to 1am ON – 1pm OFF. Mice were kept on this 7-hour advanced LD cycle until stable entrainment was achieved for at least 10-15 days. Grey shading depicts time when lights are ON.

B) Quantification of the rate of entrainment measured as the mean cumulative phase shift per day to the advanced LD cycle. (n=11-20 mice/genotype; error bars represent standard error mean; # (GRK2 Het), * (GRK2 KO), ###/**p≤0.01 vs WT, ###/*** p≤0.001 vs WT).

C) Quantification of the total days required for mice to entrain their locomotor activity rhythms to the new 7 hr advanced LD cycle. (n=11-20 mice/genotype; error bars represent standard error mean; ***p≤0.001 vs WT).

D) Quantification of the magnitude of the first day phase shift of mice in the new 7 hr advanced LD cycle. (n=11-20 mice/genotype; error bars represent standard error mean; **p≤0.01 vs WT, ***p≤0.001 vs WT).
3.3.2 **Photic Entrainment: Discrete Light Pulses:** GRK2 Mutant Mice exhibit Altered Responses to a Discrete Light Pulse during the Early and Late Subjective Night

At the behavioral level it is known that a LP exerts a resetting effect on the SCN clock in a phase-dependent manner. More specifically, the SCN clock and downstream behavioral rhythms are reset by undergoing a phase delay in response to light exposure during the early night and a phase advance in response to light exposure during the late night. In order to examine the roles of GRK2 in the process of photic entrainment to discrete light pulses administered during the subjective night, stably entrained WT and GRK2 mutant mice were released in constant darkness for 10-14 days, and given a short 15 minute LP either during the early night (CT 15, 30 lux) or late night (CT 22, 40 lux) (Fig 6). Phase shifts in response to the LP were measured by fitting regression lines through the daily activity onsets before and after the light pulse. The displacement between the two regression lines is a measure of the phase shift. In this regard, it was found that GRK2 mutant mice exhibited significantly enhanced phase delays in response to an early night LP (CT 15) as compared to WT mice (Fig 6). Conversely, GRK2 mutant mice showed a slightly but not significantly dampened phase advance in response to a late night LP as compared to WT mice (CT 22) (Fig 6).
Figure 6. Photic Entrainment to discrete light pulses

A,B) Representative double plotted actograms of WT and GRK2 mutant mice free running in constant darkness. Red dots represent time of 15-minute light pulse administration at either CT 15 or CT 22, where CT 12 represents activity onset. Grey shading depicts periods of darkness.

C,D) Quantification of the CT 15 phase delays and CT 22 phase advances in WT and GRK2 mutant mice. A regression line (red: pre LP) was fitted through the activity onsets of the final >10 days prior to light pulse administration to obtain a stable free running period. Another regression line (green: post LP) was fitted after the light pulse. The displacement between the two regression lines represents the phase shift in response to the light pulse. (n=12-21 mice/genotype; error bars represent standard error mean; ***p ≤ 0.001 vs WT).
In constant darkness, nocturnal mice are known to exhibit a free-running circadian period of locomotor activity rhythms that is slightly less than 24 hours. This periodicity in locomotor activity rhythms is endogenously generated and regulated by the core clock mechanism. In order to examine the roles of GRK2 in regulation of the core clock controlled circadian locomotor activity rhythms, WT and GRK2 mutant mice were placed in constant darkness and the free-running endogenous period was examined (Fig 7). The endogenous period was measured using at least 14 days in constant darkness by drawing a regression line through daily activity onsets. In constant darkness, the circadian clock of GRK2 mutant mice was found to be functioning at a slower pace. The resulting free-running endogenous period of locomotor activity rhythms was found to be significantly longer in GRK2 mutant mice as compared to WT mice (Fig 7).
**Figure 7. Free Running Endogenous Period**

A) Representative double plotted actograms of WT and GRK2 mutant mice free-running in constant darkness following stable entrainment to a 12:12 hr LD cycle. Grey shading depicts time when lights are ON.

B) Quantification of the endogenous free running period of locomotor activity rhythms in WT and GRK2 mutant mice. (n=12-20 mice/genotype; error bars represent standard error mean; ***p≤0.001 vs WT).
Table 2. Summary of behavioral analysis conducted on WT, GRK2 +/- and GRK2<sup>flox/flox</sup> Vgat-ires-Cre<sup>+</sup>.
Significantly different values vs. WT mice are highlighted in red. Sample sizes presented in brackets for each genotype.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>grk2+/−</th>
<th>Vgat&lt;sup&gt;−ires-Cre&lt;/sup&gt;, grk2&lt;sup&gt;flox/flox&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period (h)</td>
<td>23.6±0.03 (20)</td>
<td>23.85±0.04 (14)</td>
<td>23.85±0.04 (12)</td>
</tr>
<tr>
<td>First Day shift into 7 hr advanced LD cycle (h)</td>
<td>0.29±0.11 (20)</td>
<td>-0.53±0.15 (14)</td>
<td>-0.34±0.16 (11)</td>
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<tr>
<td>Days to re-entrain to new 7 hr advanced LD cycle</td>
<td>6.48±0.30 (20)</td>
<td>9.92±80 (14)</td>
<td>9.83±66 (11)</td>
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<tr>
<td>CT 15 Delay - 30 lux (h)</td>
<td>-1.89±.012 (20)</td>
<td>-2.35±0.10 (12)</td>
<td>-2.24±0.11 (14)</td>
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<tr>
<td>CT 22 Advance -40 lux (h)</td>
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<td>0.35±0.17 (14)</td>
<td>0.34±0.15 (12)</td>
</tr>
<tr>
<td>Phase Angle (h)</td>
<td>0.03±0.03 (21)</td>
<td>0.17±0.04 (14)</td>
<td>0.20±0.05 (12)</td>
</tr>
</tbody>
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3.4 GRK2 and Photic Entrainment at the Molecular level

It is known that at the molecular level, a light pulse exerts a resetting effect on the SCN in a phase-dependent manner. The SCN clock is reset by undergoing a phase delay in response to light exposure during the early night and a phase advance in response to light exposure during the late night, both of which result in MAPK/ERK signaling activation along with downstream CREB/CRE regulated transcriptional activation of clock genes such as per1 following light stimulation.

3.4.1 GRK2 and Light-Induced MAPK/ERK signaling

MAPK/ERK signaling activation and its regulation via additional proteins and kinases such as RKIP has been shown to be critical for regulating the process of coupling of light to clock resetting. Since the behavioral data suggested possible roles of GRK2 in the regulation of photic entrainment, it is possible that light-induced MAPK/ERK activation is also affected in the absence of GRK2. To test this hypothesis, WT and GRK2 KO mice kept in constant dark conditions for 2 consecutive days, received a 15 min light pulse at 80 lux at CT 6, CT 15, or CT22, placed back in DD for 15 minutes and killed thereafter for tissue harvest (Fig 8). Immunohistochemistry was conducted to examine the activation of the terminal kinase in the MAPK/ERK signaling cascade, p-ERK1/2, within the SCN of light pulsed and constant dark control (no light pulse) mice (Fig 8). Based on the augmented behavioral phase delays, as expected, it was established that a 15 min LP during the early night (CT15) resulted in significantly enhanced light induced MAPK/ERK activation in GRK2 KO mice as compared to the light induced MAPK/ERK activation in WT mice (Fig 8). However, unlike at the behavioral level where no significant differences were observed following a late night LP, at the molecular level, a late night LP (CT22) also resulted in significantly enhanced light-induced MAPK/ERK activation in GRK2 KO mice as compared to WT mice (Fig 8). Finally, based on the fact that light input during the subjective daytime is gated, a LP administered during the daytime failed to induce MAPK/ERK signaling to any significant extent in both WT and GRK2 KO mice (Fig 8).
Figure 8. Light Induced MAPK/ERK Signaling

A) Representative micrographs illustrating light-induced MAPK/ERK signaling activation within the SCN, measured as phospho-ERK1/2 immunoreactivity, in WT and GRK2 KO mice during the subjective early night (CT 15), subjective late night (CT 22) and subjective day (CT 6). Mice were light pulsed (15 min, 80 lux) during the indicated circadian time (CT) points, returned to DD and sacrificed 15 min following cessation of the LP. Control (DD) mice were sacrificed without receiving a light pulse at the appropriate CT.

B,C,D) Quantitation of normalized p-ERK1/2 expression in the core SCN of light pulsed and control (DD) WT and GRK2 KO mice. Values quantified as mean grey intensity for each bilateral SCN normalized to background staining from adjacent non-immunoreactive hypothalamic regions. (n=3-4 mice /genotype/treatment; error bars represent standard error mean; **p≤0.01 vs WT).
3.4.2 GRK2 and Light-Induced \textit{mper1} Transcriptional Activation

Based on the fact that light-induced MAPK/ERK signaling activation has been shown to activate CRE/CREB mediated transcriptional activation of clock genes within the SCN, the potential roles of GRK2 in the regulation of photic entrainment during the night time at the transcriptional level of \textit{mper1} was examined (Fig 9). \textit{mPER1::VENUS} transgenic mice were bred onto a GRK2 +/- background. These mice express the VENUS protein under the control of an \textit{mper1} promoter, making it possible to study the effects of reduced GRK2 expression on the transcriptional regulation of \textit{mper1}. Mice were kept in constant dark conditions for 2 consecutive days, received a 15 min light pulse at CT 15 (80 lux), were returned to DD for 4 hours following the LP, and were killed at CT 19 for tissue harvest (Fig 9). Immunofluorescence was conducted in order to examine the expression of Venus within the SCN of light pulsed and dark control (no light pulse) mice (Fig 9). The magnitude of light-induced \textit{mper1} transcriptional activation in GRK2 +/- mice was significantly augmented as compared to the light-induced activation in WT mice, whereas levels of Venus remained relatively unaltered between both genotypes under basal conditions (Fig 9). These results suggest a negative modulatory role of GRK2 in the transcriptional regulation of light-induced \textit{mper1} activation.
Figure 9. Light-induced mper1 transcription activation

A) Representative micrographs of light-induced mPER1::VENUS expression in the SCN of WT and GRK2 +/- mice. Control (DD) mice were killed without receiving a light pulse at the appropriate CT.

B) Quantitation of normalized mPER1::VENUS expression in the core SCN of light pulsed and control (DD) WT and GRK2 +/- mice. Values quantified as mean grey intensity for each bilateral SCN (in arbitrary units) ± SEM normalized to background staining from adjacent non-immunoreactive hypothalamic regions. (n=3-4 mice/genotype/treatment; error bars represent standard error mean; **p≤0.01 vs WT)
3.5 GRK2 and Circadian Core Clock Regulation

Since mutations of clock genes often result in the production of an abnormal free-running circadian period at behavioral level\textsuperscript{22-24}, in order to see if the observed behavioral phenotype of period lengthening within GRK2 mutant mice has a molecular basis within the core clock machinery, using immunohistochemistry, clock protein levels of PER1 and PER2 were examined across the 24-hour circadian cycle. PER1 protein expression is known to peak during the early night hours (CT 12-14) (Fig 10), while PER2 protein levels peak later during the subjective night around CT16-CT18 (Fig 11). GRK2 KO mice exhibited significantly augmented PER1 (Fig 10) and PER2 (Fig 11) peak expression as compared to WT mice at CT 14 and CT 18, respectively, with no detectable change in the phase of protein expression.
Figure 10. PER1 Circadian Expression in the SCN.

A) Representative micrographs of free-running endogenous PER1 rhythmic expression profiles in the SCN of WT and GRK2 KO mice placed in constant darkness for 2 days and killed at the indicated CT.

B) Quantification of the circadian expression of PER1 measured as the number of cells at a fixed threshold throughout the SCN of WT and GRK2 KO mice across a 24 hr period. (n=3-4 mice/genotype/time point; error bars represent standard error mean; *p≤0.05 vs WT).
Figure 11. PER2 Circadian Expression in the SCN.

A) Representative micrographs of free-running endogenous PER2 rhythmic expression profiles in the SCN of WT and GRK2 KO mice placed in constant darkness for 2 days and killed at the indicated CT.

B) Quantification of the circadian expression of PER2 measured as the number of cells at a fixed threshold throughout the SCN of WT and GRK2 KO mice across a 24 hr period. (n=3-4 mice/genotype/time point; error bars represent standard error mean; **p≤0.01, *p≤0.05 vs WT).
3.5.1 GRK2 and Localization of Circadian Core Clock Proteins

It is known that CK1 and GSK3 proteins can interact with PER and CRY, resulting in their phosphorylation and consequent degradation and affecting their nucleo-cytoplasmic translocation dynamics. Based on the fact that in the absence of GRK2 the amplitude of PER1 and PER2 circadian expression is elevated, in order to test the possibility that GRK2 is involved in the regulation of a mechanism coupling to core clock protein expression and localization, a complementary in vitro approach was taken to examine how GRK2 influences the nucleo-cytoplasmic localization and expression of clock proteins. Briefly, Neuro2A cells were transfected with the clock proteins tagged with a v5 epitope in conjunction with a control pcDNA 3.1 Hygro construct or a GRK2 wild type overexpression construct. From these experiments, it was found that overexpressing GRK2 significantly decreased the proportion of cells that expressed PER specifically within the nucleus, and decreased the overall abundance of PER protein within the nuclear compartment, as measured by mean fluorescence intensity (Fig 12). Interestingly, the effects of GRK2 were specific to the PER proteins, as CRY1 and CRY2 expression and localization were not affected by GRK2 overexpression (Fig 13).
Figure 12. PER Protein Expression and Localization

A) Representative micrographs illustrating PER1 and PER2 expression and localization in Neuro2A cells transfected with either pcDNA3.1 control or GRK2 WT constructs.

B) Graphs illustrating localization of PER1 and PER2 immunoreactivity in Neuro2A cells transfected with either pcDNA3.1 control or GRK2 WT constructs. Percentage of cells that exhibited PER1/PER2 staining primarily in the nucleus, or in both the nucleus and cytoplasm, is reported. (n=20-40 cells per experiment; 3 replicate experiments per condition; error bars represent standard error mean; **p≤0.01 vs pcDNA nuclear PER expressing transfected cells).

C) Graphs illustrating expression of PER1 and PER2 immunoreactivity in Neuro2A cells transfected with either pcDNA3.1 control or GRK2 WT constructs. Fluorescence intensity of PER1 and PER2 immunoreactivity was calculated within the nucleus and the entire cell of control and GRK2 overexpressing cells and normalized to background fluorescence. (n=40-150 cells; 3 replicate experiments per condition; error bars represent standard error mean; ***p≤0.001 vs pcDNA nuclear PER expressing transfected cells).
Figure 13. CRY Protein Expression and Localization

A) Representative micrographs illustrating CRY1 and CRY2 expression and localization in Neuro2A cells transfected with either pcDNA3.1 control or GRK2 WT constructs.

B) Graphs illustrating localization of CRY1 and CRY2 immunoreactivity in Neuro2A cells transfected with either pcDNA3.1 control or GRK2 WT constructs. Percentage of cells that exhibited CRY1/CRY2 staining primarily in the nucleus, or in both the nucleus and the cytoplasm, is reported. (n=20-40 cells per experiment; 3 replicate experiments per condition; error bars represent standard error mean).

C) Graphs illustrating expression of CRY1 and CRY2 immunoreactivity in Neuro2A cells transfected with either pcDNA3.1 control or GRK2 WT constructs. Fluorescence intensity of CRY1 and CRY2 immunoreactivity was calculated within the nucleus of control cells and cells overexpressing GRK2 and normalized to background fluorescence. (40-150 cells; 3 replicate experiments per condition; error bars represent standard error mean).
3.5.2 Potential Role of GRK2 in CK1 mediated PER localization

To assess CK1 as a potential mechanism by which GRK2 might regulate PER protein localization, the effects of the CK1 inhibitor, PF-670462, on PER localization were examined in the context of GRK2 overexpression. Briefly, Neuro2A cells were transfected with a V5-tagged PER1 construct in conjunction with a control pcDNA 3.1 Hygro construct or a GRK2 wild type overexpression construct, and 24 hours later were treated with PF-670462 for 4 hours. In agreement with previous experiments it was found that overexpressing GRK2 significantly decreased the number of cells expressing PER1 within the nucleus and decreased the overall abundance of PER1 protein specifically within the nuclear compartment (Fig 14). However, treating GRK2 overexpressing cells with PF-670462 significantly increased both the number of cells expressing PER1 within the nucleus and the level of PER1 expression within the nuclear compartment (Fig 14). These results suggest that GRK2 is potentially involved in the regulation of PER protein localization via a CK1-dependent mechanism.
A

pcDNA

GRK2

pcDNA

GRK2

Basal

PF-670462

B

C

GRK2

DAPI

% PER1 vs Control

pcDNA GRK2 WT pcDNA GRK2 WT

PER1 Fluorescence Intensity

pcDNA GRK2 WT pcDNA GRK2 WT

Basal PF-670462

***
Figure 14. Effect of CK1 on PER1 localization

A) Representative micrographs illustrating PER1 expression and localization in Neuro2A cells transfected with either pcDNA3.1 control or GRK2 WT constructs and treated with the CK1 inhibitor, PF-670462.

B) Graphs illustrating localization of PER1 immunoreactivity in Neuro2A cells transfected with either pcDNA3.1 control or GRK2 WT constructs under basal conditions and following a 4 hr exposure to PF-670462. Percentage of cells that exhibited PER1 staining primarily in the nucleus, or in both the nucleus and cytoplasm, is reported. (n=20-40 cells per experiment; 3 replicate experiments per condition; error bars represent standard error mean; ***p≤0.001 vs Basal GRK2 WT nuclear PER expressing transfected cells).

C) Graphs illustrating expression of PER1 immunoreactivity in Neuro2A cells transfected with either pcDNA3.1 control or GRK2 WT constructs under basal conditions and following a 4 hr exposure to PF-670462. Here, fluorescence intensity of PER1 and PER1 immunoreactivity was calculated within the nucleus and the entire cell of control and GRK2 overexpressing cells and normalized to background fluorescence under the specified conditions. (40-150 cells; 3 replicate experiments per condition; error bars represent standard error mean; ***p≤0.001 vs Basal GRK2 WT nuclear PER expressing transfected cells).
3.6 GRK2 and GPCR Regulation

3.6.1 GRK2 mediated VPAC2 and V1bR Regulation in Neuro2A Cells

*In vitro* and *in vivo* experiments were conducted in order to gain insight on how GRK2 is affecting GPCR signaling within the SCN. Here the primary goal was to examine how GRK2 influences ligand-induced internalization and recycling of some GPCRs involved in circadian clock function. In these studies, VIP/PACAP preferring type 2 receptor (VPAC2) and arginine vasopressin (AVP) receptor (V1b) were examined, as they have previously been shown to play a role within the SCN [47-51]. Briefly, Neuro2A cells were transfected with one of the following plasmids—1) pcDNA 3.1 Hygro, 2) GRK2 wild type overexpression construct, or 3) GRK2 K220R – dominant negative kinase dead GRK2 mutant construct—in conjunction with either 1) VPAC2-GFP or 2) V1b-YFP receptor constructs. 18-24 hr following transfection, VPAC2 and V1b transfected cells were treated for 20 minutes at 37°C with VIP (1µM) and AVP (100nM), respectively, in order to induce receptor activation and consequent internalization. Basal receptor states were examined without any ligand addition. Immunocytochemistry was used to qualitatively examine and compare receptor localization for each condition between pcDNA control cells, GRK2 WT overexpressing and GRK2 K220R kinase-dead overexpressing mutant cells. Basally in pcDNA control cells, GRK2 WT overexpressing and GRK2 K220R overexpressing cells, both VPAC2 and V1b were primarily localized within the plasma membrane and uniformly within the cytoplasm, with minimal endosomal or punctate (dot-like) cytoplasmic staining (Fig 15, Fig 16). Application of VIP or AVP successfully induced VPAC2 and V1b receptor internalization, respectively, in both pcDNA control and GRK2 overexpressing cells, as seen by a sharp increase in endosomal or punctate (dot-like) cytoplasmic staining and a strong decrease in plasma membrane staining (Fig 15, Fig 16). However, VPAC2 receptor internalization was severely blunted in GRK2 K220R overexpression, whereas the negative effect of GRK2 K220R on V1b receptor internalization was more moderate (Fig 15, Fig 16). In the case of VPAC2, once the GPCR is activated and GRK2 is recruited to the receptor complex, the kinase dead GRK2 cannot phosphorylate the GPCR and in turn fails to catalyze the GPCR-β-arrestin interaction that is required to initiate receptor internalization.
Figure 15. GRK2 and VPAC2 Regulation

Representative micrographs illustrating VPAC2-GFP receptor expression in pcDNA3.1 control, GRK2 WT, and GRK2 K220R transfected Neuro2A cells. VPAC2 transfected cells were incubated with VIP for 20 mins to induce receptor internalization (+ VIP). Receptor internalization can be visualized by the appearance of dot/punctate cytoplasmic staining as compared to the diffuse cytoplasmic and plasma membrane-localized staining in the basal, unstimulated state (-VIP). Experiments were repeated twice to ensure validity and reproducibility. White arrows illustrate plasma membrane-bound VPAC2 expression pattern. Red arrows illustrate internalization and endosome-localized VPAC2 expression.
Figure 16. GRK2 and V1bR Regulation

Representative micrographs illustrating V1b-YFP receptor expression in pcDNA3.1 control, GRK2 WT, and GRK2 K220R transfected Neuro2A cells. V1b transfected cells were incubated with AVP for 20 mins to induce receptor internalization (+AVP). Receptor internalization can be visualized by the appearance of dot/punctate cytoplasmic staining as compared to the plasma membrane-localized staining in the basal, unstimulated state (-AVP). Experiments were repeated three times to ensure validity and reproducibility. White arrows illustrate plasma membrane-bound V1b expression pattern. Red arrows illustrate internalization and endosome-localized V1b expression.
3.6.2 GRK2 mediated VPAC2 Regulation in the SCN

Previous research has shown that VPAC2 protein is rhythmically expressed within the murine SCN [48]. Based on the fact that in vitro, the lack of GRK2 activity abolishes VIP-mediated VPAC2 internalization and consequently its degradation, it can be hypothesized that post-translational events such as GRK2-mediated receptor phosphorylation, internalization, recycling and degradation might be contributing to the generation of rhythmic expression within the SCN. To test this possibility, WT and GRK2 knockout mice were killed at 4-h intervals across a 24-hour period and VPAC2 protein expression within the SCN was examined using immunohistochemistry (Fig 17). In WT mice VPAC2 expression was found to be rhythmic with levels peaking around the late night (CT 18). In the absence of GRK2, VPAC2 expression retained its circadian rhythmicity; however, levels were significantly augmented during the early and mid-night (CT14/CT18) and throughout the subjective day (CT 2/CT10). These results in conjunction with the in vitro data showing the involvement of GRK2 in the trafficking of VPAC2, suggests a potential modulatory role of GRK2 on the expression of the receptor within the SCN and ultimately on how the SCN clock is responsive to and coupling to stimuli during different times of the day.
Figure 17. GRK2 and VPAC2 Expression within the SCN

A) Representative micrographs of free-running endogenous VPAC2 rhythmic expression profiles in the SCN of WT and GRK2 KO mice placed in constant darkness for 2 days and killed at the indicated CT.

B) Quantification of circadian expression of VPAC2 across a 24 hr period in the SCN of WT and GRK2 KO mice. Values quantified as mean grey intensity for each bilateral SCN normalized to background staining from adjacent non-immunoreactive hypothalamic regions. (n=3 mice/genotype/time point); error bars represent standard error mean; *p≤0.05 vs WT).
4 Discussion

GRK2 is a critical component in the regulation of GPCR signaling and is involved in a myriad of cellular contexts and processes. However, its contribution in the regulation of the circadian clock has not been investigated to date. These studies collectively serve to examine the roles of GRK2 in the context of mammalian circadian clock functioning at the behavioral and molecular levels. The absence of GRK2 in mice was found to significantly lengthen the circadian period of locomotor activity rhythms and delay the process of photic entrainment to a 7 hr advance in the LD cycle, while slightly dampening the phase advancing response to a light pulse during the late night. However, the lack of GRK2 markedly augmented the phase delaying response to a light pulse during the early night. At the molecular level, the lack of GRK2 within the SCN significantly augmented light-induced MAPK/ERK signaling during the early and late night and significantly enhanced light-induced transcriptional activation of mper1 during the early night.

Furthermore, the lack of GRK2 resulted in enhanced PER1 and PER2 expression within the SCN during the night, while in vitro studies showed a CK1-dependent regulatory role of GRK2 on PER protein localization in Neuro2A cells. Lastly, GRK2 activity was found to be necessary for VIP/PACAP- induced activation and internalization of VPAC2 receptor. As a result, in the absence of GRK2, VPAC2 circadian rhythmic expression within the SCN was significantly augmented throughout the 24-h cycle. Strengthening the validity of these conclusions, completely ablating GRK2 levels and partially reducing GRK2 levels using two distinct genetic approaches in mice both resulted in complementary observations at the molecular and behavioral levels.

The importance of MAPK/ERK signaling activation and regulation as a marker for light-induced clock resetting has been proposed in numerous studies. Light-induced MAPK/ERK activation in the SCN mediates behavioral clock resetting via numerous mechanisms such as activation of CREB, and transcription of immediate early genes (eg. c-fos) and clock genes (e.g. per). Studies have shown that PKC-mediated Raf kinase inhibitor protein (RKIP) phosphorylation regulates MAPK/ERK signaling and plays a role in mediating photic entrainment within the murine SCN. More specifically, it has been shown that unphosphorylated RKIP binds to and inhibits Raf1, such that Raf1 is then prevented from phosphorylating MEK and activating downstream MAPK/ERK signaling components. Light activates PKC and induces its phosphorylation of RKIP. RKIP phosphorylation results in
dissociation of RKIP and Raf1. Consequently, RKIP dimerization converts RKIP from a Raf1 inhibitor to a G-protein coupled receptor kinase-2 (GRK2) inhibitor. Here GRK2 can be hypothesized to negatively regulate GPCR-associated signaling, resulting in MAPK/ERK activation. In the absence of GRK2, it was found that an early night LP augmented behavioral phase delays and enhanced light-induced MAPK/ERK signaling and *per1* transcriptional activation. Overall, these results suggest a negative modulatory role of GRK2 at the molecular level in the process of photic entrainment, by regulating the signaling pathways that ultimately impinge on MAPK/ERK activation and clock gene transcription. From these results, it can also be concluded that the capacity of the circadian clock to accurately gate and respond to light during different phases of the circadian cycle is not altered in mice lacking GRK2. Research has shown that a light stimulus can have differing effects at the molecular signaling level depending on the time of the night it is administered. During the early night, a light pulse activates calcium/nitric oxide mediated signaling, resulting in MAPK/ERK signaling activation, CREB/CRE mediated transcriptional activation and a PER2-dependent phase delay at the behavioral level. During the late night, a light pulse activates a cGMP/Protein Kinase G dependent, ryanodine receptor independent signaling, resulting in MAPK/ERK signaling activation, CREB/CRE mediated transcriptional activation and phase advances at the behavioral level. Furthermore, the activation of cAMP/PKA has been found to enhance light/glutamate signaling events and clock state resetting during early night, while antagonizing light/glutamate-induced clock resetting in the late night. In this regard, GRK2 might serve to limit or desensitize cAMP/PKA mediated signaling during the early night, allowing for enhanced MAPK/ERK signaling and augmented behavioral phase delays in the absence of GRK2. In contrast, during the late night, the absence of GRK2 is predicted to enhance cAMP/PKA signaling, which in turn opposes light/glutamate-induced clock resetting, thereby dampening late-night phase advances. Of note, the dissociative effect observed between the significantly enhanced late night LP-induced MAPK/ERK signaling activation and slightly diminished late night LP-induced behavioral phase advances in GRK2 mutant mice is puzzling and deserves future review and attention.

Another possible avenue of photic entrainment that is MAPK/ERK-dependent but CREB-independent is mTOR (mammalian target of rapamycin) pathway-mediated control of protein translation. mTOR is a Ser/Thr kinase that serves as the central component of the multi-
protein mTOR complex 1 (mTORC1). The mTOR pathway serves as a major regulator of ribosomal protein synthesis. mTORC1 consist of two signaling arms, both of which are acutely triggered by light within the SCN. One arm is activated by p70 S6 kinase (p70 S6K), consisting of the S6 ribosomal protein resulting in the activation of the translation of mRNAs by increasing ribosomal activity. The second arm is activated by eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) inhibition that is involved in the activation of eIF4E cap-dependent translation initiation.

Light has been found to trigger the phase-dependent activation of the mammalian target of rapamycin (mTOR) signaling pathway via the activation of the RSK complex, consisting of either the p70 S6K (S6 ribosomal protein kinase – S6K) or the p90 RSK (MAPK-activated protein kinase-1) that phosphorylates ribosomal S6 at serine 240/244 or 235/236, respectively. Of particular interest, within the SCN, an in vivo infusion of the mTOR inhibitor rapamycin has been shown to significantly reduce light-evoked S6 phosphorylation (a marker of mTOR activity), PER expression, and early night phase delays, while promoting late night phase advances. The effects of rapamycin on light-induced clock gene expression and phase shifts are opposite to what are observed in the absence of GRK2. It would be interesting to examine if GRK2 is involved in the translational control of clock proteins via the mTOR pathway, instead of or in conjunction with regulating the MAPK/ERK/CREB-dependent transcription of clock genes.

An abrupt 7hr advance in the LD cycle (eastward jetlag) results in locomotor activity rhythms that gradually advance (they start running earlier each day) until their activity onset or their period of activity is entrained to the new environmental LD cycle. This process takes a few days to occur as the internal clock is undergoing daily sequential and additive phase adjustments (shifts) along with gradual changes in the circadian clock period to adjust to the new LD cycle. Mice lacking GRK2 were found to be significantly slower and took significantly longer to re-entrain their locomotor activity rhythms to a new 7 hr advanced LD cycle. Similar to their response to a late night light pulse, GRK2 mutant mice exhibited smaller daily phase advances when transferred to a new 7 hr advanced LD cycle. Interestingly, these animals initially phase delayed (rather than phase advanced) when placed into the new advanced LD cycle. These results, in combination with the late night light pulse behavioral data suggest that the lack of
GRK2 alters the ability of the SCN clock to couple light to molecular and behavioral clock resetting.

The period of free-running locomotor activity rhythms in mice lacking GRK2 was significantly lengthened. Research has shown that clock genes per1 and per2 play a critical role in regulating period length. It has been established that mice lacking the per1 and or the per2 gene exhibit shorter free-running periods at the behavioral locomotor activity level, and, in the case of per2 knockouts, eventually become arrhythmic. Conversely, overexpression of per1 has been shown to result in an increase in period length\textsuperscript{25}. Consistent with previous evidence suggesting the influence of PER1 and PER2 on regulating the period of behavioral rhythms, it was found that mice lacking GRK2 exhibited a significant increase in PER1 and PER2 amplitude. These data suggest that GRK2 potentially serves to regulate the core clock mechanism within the SCN by mediating the expression of the endogenous clock at the transcriptional and/or translational level.

Casein kinase 1 delta (ck1δ) is a kinase responsible for regulating the 24 hour period and pace of the circadian clock by phosphorylating PER, delaying its accumulation and nuclear entry, and consequently increasing its turnover via the ubiquitin-proteasome pathway\textsuperscript{16,17}. An \textit{in vitro} approach was utilized to examine if the changes in PER expression in mice lacking GRK2 are a result of changes in the subcellular localization of PER. It was found that overexpressing GRK2 in Neuro2A cells significantly decreased the number of cells expressing PER specifically within the nucleus and decreased the expression of PER protein specifically within the nuclear compartment, measured as fluorescence intensity. Interestingly, CRY1 and CRY2 expression and localization was not significantly affected by GRK2 overexpression. CK1 and other kinases such as Glycogen Synthase Kinase 3 (GSK3) have been shown to phosphorylate and result in the translocation and consequent degradation of PER proteins. The phosphorylation of PER, however, has site-specific effects at the clock level\textsuperscript{18}. For instance, phosphorylation of different sites on PER by casein kinase 1 (CK1) and glycogen synthase kinase 3 can lead to opposite effects on PER stability and on the circadian period\textsuperscript{18}. More specifically, phosphorylation at one site enhances the rate of degradation of PER and decreases the circadian period, while phosphorylation at another site stabilizes PER, enhances the transcription of the \textit{per} gene, and increases the circadian period\textsuperscript{18}. The roles of GSK3 in circadian clock regulation are also becoming increasingly appreciated. Similar to the effects of GRK2 ablation, blocking GSK3 using lithium salt has been shown to result in period lengthening and an increase in clock gene
transcription, allowing for increased amplitude and synchrony of clock gene oscillations\textsuperscript{21}. To assess the potential involvement of CK1 in GRK2-regulated PER protein localization, we tested the effects of the CK1 inhibitor PF-670462 on PER localization. Treating GRK2 overexpressing cells with PF-670462 resulted in a significant increase in both the number of cells expressing PER1 within the nucleus and the overall abundance of PER1 protein within the nuclear compartment. In mice lacking GRK2, PER nuclear expression was found to be elevated during the night time, resulting in an increase in period length of locomotor activity rhythms. In accordance with previous experiments using PF-670462 in mice, it was also found that blocking CK1 significantly increased nuclear PER retention, inhibited PER degradation and lengthened the period\textsuperscript{18-20}. Even though GRK2 seems to be acting via a CK1-dependent mechanism in regulating the localization of PER proteins, the stability of PER proteins does not seem to be affected by GRK2 as illustrated by the fact that total levels of the PER1 within the entire cell remain unaffected by overexpressing GRK2. These results suggest that GRK2 is potentially involved in the regulation of PER protein localization via a modulatory role on a CK1-dependent mechanism. Future work in this regard should examine how GRK2 is specifically acting to regulate CK1-dependent signaling. Furthermore, it would be interesting to examine if additional kinase signaling pathways such as GSK3 are also affected by GRK2 mediated signaling regulation. Such data could potentially help explain the lack of effects of GRK2 at the protein stability level via a differential PER phosphorylation site-specific effect on the circadian clock\textsuperscript{18}.

\textit{In vitro} studies revealed that GRK2 is responsible for regulating AVP- and VIP/PACAP-induced V1b and VPAC2 receptor internalization, respectively. A kinase-dead version of GRK2 blocked AVP- and VIP-induced internalization of their cognate receptors, V1b and VPAC2, respectively, and presumably failed to diminish receptor desensitization. VPAC2 receptor internalization was severely blunted by GRK2 K220R overexpression, whereas the effect of GRK2 K220R on V1b receptor was more subtle. In the case of VPAC2, once the GPCR is activated and GRK2 is recruited to the receptor complex, the kinase dead GRK2 cannot phosphorylate the GPCR and in turn fails to catalyze the GPCR-\(\beta\)-arrestin interaction that is required to initiate receptor internalization. Interestingly, previous research has similarly shown that GRK2 K220R overexpression in smooth muscle cells significantly diminishes VIP-induced phosphorylation, internalization and degradation of VPAC2 receptors, whereas GRK2 WT overexpression results
in a dose-dependent increase in VIP-induced VPAC2 phosphorylation, internalization and recycling/degradation

Previous research has shown that VPAC2 protein is rhythmically expressed within the murine SCN. Based on the fact that in vitro, the lack of GRK2 abolishes VIP-mediated VPAC2 internalization and consequently its degradation, post-translational events such as GRK2-mediated receptor phosphorylation, internalization, recycling and degradation might be contributing to the generation of rhythmic VPAC2 expression within the SCN. Based on the fact that GRK2 is required to initiate receptor internalization and consequent receptor degradation of VPAC2, in the absence of GRK2, the lack of receptor internalization and downstream degradation could potentially explain the fact that amplitude of VPAC2 circadian expression in mice lacking GRK2 was found to be significantly elevated across the 24h cycle as compared to WT mice. These results in conjunction with the in vitro data showing the requirement of GRK2 in the internalization and degradation of VPAC2 and the in vivo augmented behavioral and molecular responses observed in response to an early night light pulse, suggest a potential modulatory role of GRK2 on the expression of VPAC2-mediated signaling within the SCN and ultimately on how the SCN clock is responsive during the early night. Literature classifies GPCRs at either Class A or Class B, depending on the β-arrestin-receptor complex stability. Class A GPCR typically form unstable complex with β-arrestin and as a result are rapidly recycled, whereas Class B GPCRs form stable complex with β-arrestin within endosomes, allowing them to be retained within the cell for prolonged periods of time. While V1b is classified as a Class A GPCR, VIP/PACAP VPAC2 is classified as a Class B receptor. As a Class B GPCR, VPAC2 is conceivably more prone to endosomal degradation following internalization. One possibility is that GRK2 is involved in the internalization and turnover of VPAC2 receptors within the SCN.

VPAC2-deficient mice display severe disruptions of circadian behavior and clock gene oscillations. Studies using these mice have shown that the VPAC2 receptor is essential for the expression of robust circadian rhythmicity. VPAC2-deficient mice exhibit a complete lack of coordinated rhythmic clock gene expression in the SCN along with the lack of rhythmic expression of clock-controlled genes such as avp. Behaviorally, the lack of coordinated clock gene expression in VPAC2-null mice results in weakly rhythmic locomotor activity, with no
clear time of activity onset under free running conditions\textsuperscript{47}. Overall, based on the fact that VPAC2 signaling is crucial for the proper functioning of the circadian clock and the fact that GRK2 ablation influences the expression of VPAC2, these data suggest that GRK2 might act via VIP/PACAP-mediated VPAC2 signaling mechanism within the SCN to critically modulate photic entrainment and rhythm generation and maintenance.

The collective data suggest that VPAC2-mediated cAMP/PKA signaling within the SCN seems to be a likely candidate regulated by GRK2 within the SCN. GRK2 during the early night might serve to limit or desensitize VPAC2-coupled cAMP/PKA mediated signaling. In mice lacking GRK2, the lack of receptor desensitization and increased VPAC2 expression could potentially allow for enhanced light-induced MAPK/ERK signaling, and consequently augmented behavioral phase delays during the early night. Furthermore, PKA has been suggested to target CK1 and result in its phosphorylation at serine 370\textsuperscript{75} (Fig 18). Serine 370 phosphorylated CK1 has been shown to have lower kinase activity towards certain substrates\textsuperscript{75} (Fig 18). PER proteins are direct phosphorylation targets of CK1 and would be affected by changes in CK1 kinase activity. CK1-mediated phosphorylation of PER at specific residues can increase the rate of PER degradation and enhance PER nuclear translocation\textsuperscript{17} (Fig 18). Pharmacological inhibition of CK1 has been shown to promote nuclear PER retention, while genetic ablation of CK1 has shown to result in period elongation in mice\textsuperscript{17,20}. In the absence of GRK2, the lack of VIP-induced VPAC2 desensitization and internalization should enhance PKA/cAMP signaling activity, which in turn should inhibit CK1 activity through a PKA-dependent mechanism. In the absence of GRK2, increased PER nuclear accumulation, potentially decreased PER degradation and an increase in period length of locomotor activity rhythms have been observed, all of which are observed when there is a decrease in CK1 activity via pharmacological inhibition or genetic ablation of CK1. On the other hand, GRK2 overexpression should facilitate VIP-induced VPAC2 desensitization and internalization, resulting in a decrease in PKA/cAMP signaling activity and a consequent derepression of CK1 activity (Fig 18). Overexpressing GRK2 has been shown to result in decreased PER nuclear translocation and accumulation, both of which are characteristics of an increase in CK1 activity. Pharmacological inhibition of CK1 activity has been shown here to negate the effects of GRK2 overexpression on PER nuclear translocation and accumulation. These results further strengthen the potential association between GRK2, CK1 and PER localization and expression dynamics. To further strengthen and validate this model, future
studies should examine the changes proposed in the signaling components, including changes in cAMP/PKA activity and CK1 phosphorylation status in the presence and absence of GRK2.

To summarize, GRK2 seems to be functioning as an important modulator of the mammalian circadian clock, where it plays a critical role in setting the clock speed and in regulating photic entrainment by negative regulating the receptor mediated signaling pathways culminating in period activation and nucleo-cytoplasmic trafficking of its protein product.
VPAC$_2$ $\rightarrow$ G$_{\alpha}$ $\rightarrow$ Adenylyl Cyclase $\rightarrow$ cAMP $\rightarrow$ PKA $\rightarrow$ CK1-p $\rightarrow$ CK1 $\rightarrow$ PER-p $\rightarrow$ PER $\downarrow$ PER degradation

Homologous Desensitization
Figure 18. Model of GRK2 regulation on VPAC2 mediated PER regulation

Diagram illustrating a potential model mechanism of the role of GRK2 on VPAC2 receptor activated cAMP/PKA-signaling involved in PER regulation. GRK2-mediated phosphorylation of VPAC2 results in its homologous desensitization and internalization, thereby reducing cAMP/PKA signaling. CK1 is a substrate of PKA, such that PKA-mediated phosphorylation of CK1 results in reduced CK1 kinase activity. CK1 is known to phosphorylate PER and induce its degradation. In the absence of GRK2, VPAC2 phosphorylation, receptor internalization and cAMP/PKA signaling desensitization fail to occur. As a consequence, PKA phosphorylates CK1 at an enhanced rate. This reduction in CK1 kinase activity leads to less phosphorylation and degradation of PER1, and enhanced PER1 nuclear retention. Conversely, overexpression of GRK2 enhances VPAC2 phosphorylation, receptor internalization and cAMP/PKA signaling desensitization, culminating in reduced PKA activity. This results in lower levels of CK1 phosphorylation, increased CK1 kinase activity, and increased PER1 phosphorylation and degradation. Nuclear retention of PER1 is inhibited.
References


