RESEARCH HIGHLIGHT

GRK2: putting the brakes on the circadian clock

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G protein-coupled receptor kinases (GRKs) are a family of serine/threonine protein kinases that terminate G protein-coupled receptor (GPCR) signaling by phosphorylating the receptor and inducing its internalization. In addition to their canonical function, some GRKs can phosphorylate non-GPCR substrates and regulate GPCR signaling in a kinase-independent manner. GPCRs are abundantly expressed in the suprachiasmatic nucleus (SCN), a structure in the mammalian brain that serves as the central circadian pacemaker. Various facets of circadian timekeeping are under the influence of GPCR signaling, and thus are potential targets for GRK regulation. Despite this, little attention has been given to the role of GRKs in circadian rhythms. In this research highlight, we discuss our latest findings on the functional involvement of GRK2 in mammalian circadian timekeeping in the SCN. Using *grk2* knockout mice, we demonstrate that GRK2 is critical for maintaining proper clock speed and ensuring that the clock is appropriately synchronized to environmental light cycles. Although *grk2* deficiency expectedly alters the expression of a key GPCR in the SCN, our study also reveals that GRK2 has a more direct function that touches the heart of the circadian clock.

Keywords: G protein-coupled receptor kinase 2; G protein-coupled receptor; circadian rhythms; suprachiasmatic nucleus; PERIOD proteins; nuclear trafficking; transcription; light; phosphorylation

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Introduction

An overview of circadian clock regulation

From unicellular cyanobacteria to plants and humans, the majority of terrestrial organisms evolved a circadian timekeeping machinery that allows them to anticipate the 24h cycles of light and darkness (day vs. night) and to organize biochemical, behavioural and physiological processes accordingly [1]. Species-specific differences aside, circadian timekeeping relies on a molecular clock within single cells whose function or activity oscillates with a period of roughly 24h [1]. Cellular clocks are found in most tissues throughout an animal's body and function as peripheral oscillators to control tissue-specific rhythms; they are also found in a central pacemaking tissue (or neuronal network) that coordinates the activities of peripheral clocks and ensures their synchrony with the environmental light-dark cycle [1]. In mammals, this central pacemaker is located in the suprachiasmatic nucleus (SCN), a small region within the hypothalamus that receives direct inputs from photosensitive retinal cells [2]. As the master circadian pacemaker, the SCN

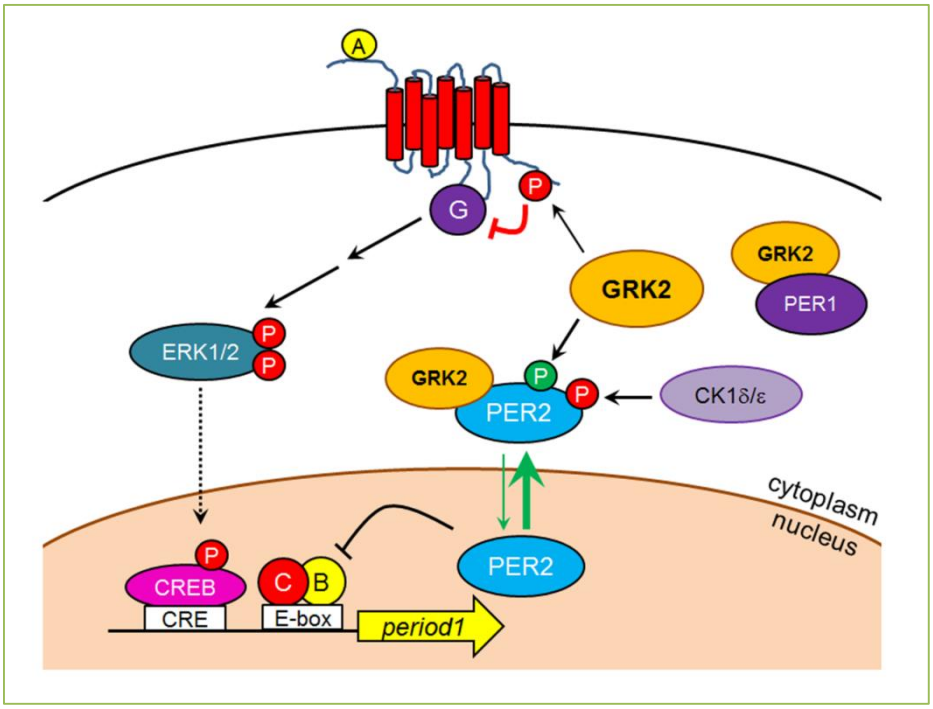

Figure 1. A proposed model for the canonical and non-canonical functions of GRK2 in the SCN pacemaker. Agonist-stimulated GPCRs activate the MAPK/ERK pathway in a G protein-dependent manner, leading to phospho-activation of CREB and CRE-mediated transcription of the *mPeriod1* gene. GRK2 acts in a canonical fashion, inhibiting this pathway by triggering the internalization of the GPCRs. Additionally, GRK2 has a non-canonical function whereby it physically associates with PER proteins and promotes the phosphorylation of PER2. We postulate that GRK2 may be a direct protein kinase for PER2. Both the physical binding and phosphorylation of PER proteins by GRK2 may mediate PER cytoplasmic retention. A, receptor agonist; B, BMAL1; C, CLOCK; G, heterotrimeric G protein; P, phosphorylation. Reprinted with permission [23].

generates self-sustained ~24h rhythms that are directly synchronized by environmental light cues [2].

The mammalian molecular clockwork, whether in central or peripheral tissues, consists of so-called "clock genes" and their protein products that, together, function within interlocked transcription-translation feedback loops (TTFLs) to drive circadian rhythms of gene expression [3]. The primary TTFL consists of CLOCK and BMAL1 heterodimers working in concert to activate the transcription of *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1*, *Cry2*) genes via binding to E-box motifs within their gene promoters [3]. Following translation, PER and CRY proteins accumulate and eventually translocate to the nucleus where they repress CLOCK-BMAL1 activity, thus shutting down their own gene expression [3]. This autorepression is relieved once PER and CRY proteins are degraded, allowing a new round of E-box-mediated transcription to ensue [3]. A time delay in one or more steps of this feedback loop is required to generate molecular rhythms with the characteristic 24h period [4]. Additionally, the phase of these molecular oscillations is responsive to environmental light cues, which can induce *Per1/2* expression through cAMP response element-binding protein (CREB)-mediated *Per1/2* transcription [5].

By delaying the feedback inhibition, various post-translational modifications (PTMs) have been shown to work hand-in-hand with TTFLs to determine the 24h period of the circadian clock [6]. PTMs such as phosphorylation, sumoylation and ubiquitination of core clock components serve to regulate their localization, degradation and activity, and thus contribute to the fine-tuning of circadian rhythms [6-8]. Of these, the most well documented clock-regulatory PTM is phosphorylation. Protein kinases such as casein kinase-1 (CK1), glycogen synthase kinase-3 (GSK3), mitogen-activated protein kinase 1/extracellular signal-regulated kinase 2 (MAPK1/ERK2), and calmodulin-dependent protein kinase II (CaMKII) have been shown to phosphorylate components of the core clock, altering their cellular localization, stability, and transcriptional activity [9-11]. Conversely, dephosphorylation by protein phosphatases such as protein phosphatase 1 (PP1), PP2A and PP5 can also regulate the function of core clock components [12]. This balance between phosphorylation and dephosphorylation sets the stage for

phosphorylation-regulated proteolysis of core clock proteins via the ubiquitin-proteasome system (UPS) [13]. The SKP1−Cullin1−F-box protein (SCF) E3 ubiquitin ligase protein complexes recognize specific phosphorylated substrates and catalyze their ubiquitination, ultimately leading to their proteasomal degradation [8, 13]. Within the SCF E3 ubiquitin ligase complex, the F-box and WD40 domain-containing protein serves as the substrate recognition subunit: in mammals, this subunit is beta-transducin repeat containing protein (βTrCP), which has been shown to target PER1 and PER2 for degradation [8]. Last but not least, sumoylation of BMAL1 by the SUMO3 ligase has been found to play a critical role in the spatiotemporal co-activation of CLOCK-BMAL1 by CREB-binding protein (CBP) [14].

Just as PTMs regulate the activity of the circadian clock, they in turn can be regulated by cues from the external environment as well as from neighbouring cells. Within the SCN, clock neurons communicate with each other and respond to changes in environmental light conditions via the activation of G protein-coupled receptors (GPCRs) by their extracellular ligands [15]. The SCN expresses a rich array of GPCRs including receptors for several neurotransmitters (e.g., glutamate, serotonin) and neuropeptides (e.g., arginine vasopressin [AVP], vasoactive intestinal peptide [VIP], pituitary adenylate cyclase activating peptide [PACAP], gastrin releasing peptide [GRP]) [2, 3, 16].

The activity of GPCRs is regulated by G protein-coupled receptor kinases (GRKs), a family of serine/theorine kinases that directly phosphorylate intracellular domains of agonist-bound GPCRs and attenuate G protein-dependent signaling via receptor desensitization and/or internalization [15, 17, 18]. In mammals, there are seven GRKs that are classified into three subfamilies based on sequence homology and regulatory mechanisms: the GRK1-like subfamily (GRK1 and GRK7), which is restricted to retinal photoreceptors; the GRK2-like subfamily (GRK2 and GRK3), which is ubiquitously expressed; and the GRK4-like subfamily (GRK4, GRK5 and GRK6) [17, 19].

Given the importance of GPCR signaling in SCN clock function, we attempted to understand the potential significance of GRKs in circadian timekeeping. We focused on GRK2, because it is robustly expressed in the SCN and it has been shown to desensitize GPCRs that are crucial for SCN clock regulation [20]. However, GRK2 possesses functions beyond that of a GPCR kinase: it can regulate cell signaling in a kinase-independent manner, and phosphorylate non-GPCR substrates such as receptor tyrosine kinases, structural proteins and intracellular signaling proteins [21, 22]. With its diverse cellular functions, GRK2 is poised to regulate the circadian clock in ways that we had not originally anticipated.

GRK2: regulator of clock speed and entrainment

In the study by Mehta et al. (2015), our lab took a comprehensive approach to investigate the contributions of GRK2 to circadian clock regulation [23]. Initial examination of *grk2* mRNA and GRK2 protein expression indicated that both are abundant in the SCN with no fluctuation throughout the circadian cycle. Circadian behaviour was assessed in two different murine *grk2*-deficient models: a conventional *grk2* heterozygous (*grk2*$^{+/-}$) strain, and a conditional Cre recombinase-mediated knock-out of *grk2* (*grk2* cKO) in GABAergic cells using the promoter for vesicular GABA transporter (*Vgat*) [24-26]. The *Vgat*-driven *grk2* cKO strain lacks GRK2 expression in virtually all SCN neurons as well as other GABAergic cells throughout the brain. Compared to wild-type controls, disruption of *grk2* by both approaches significantly delayed the onset of daily activity in a fixed 12 hour light: 12 hour dark (LD) cycle, and slowed the period of behavioural rhythms under constant darkness (DD). Under dim constant light (LL), only *grk2* cKO mice showed a longer period relative to wild-type controls. To examine the effects of *grk2* ablation on the ability of the circadian system to entrain to environmental light, we exposed mice briefly to light during either their early or late subjective night, and measured the magnitude of phase resetting by delays and advances, respectively. Compared with wild-type animals, *grk2*$^{+/-}$ and *grk2* cKO mice exhibited significantly larger phase delays to early night light stimulation. On the other hand, late night phase advances were markedly attenuated in *grk2* cKO, but not *grk2*$^{+/-}$, mice relative to wild-type controls. Under experimental jetlag conditions where the 12:12 LD cycle was abruptly advanced by 7 h, both *grk2*$^{+/-}$ and *grk2* cKO mice required more days to re-entrain to the shifted LD cycle compared with their wild-type counterparts. In summary, using behavioural paradigms we found that both the speed of the circadian clock and its ability to entrain to light are critically regulated by GRK2. The germinal loss of one copy of *grk2* slows the period of behavioural rhythms under DD, enhances acute phase delays and slows the rate of re-entrainment to an advanced LD cycle. In addition to those phenotypes, the loss of both copies of *grk2* in GABAergic neurons enhances the lengthening of behavioural rhythms under LL and attenuates the acute phase-advancing effects of a late night light pulse.

To determine whether these behavioural phenotypes had a molecular correlate at the level of clock gene expression, we went on to characterize the circadian rhythmic expression of PERIOD1 (PER1) and PERIOD2 (PER2) proteins in the SCN by immunohistochemistry. In wild-type mice, PER1 and PER2 rhythms in the SCN peak in the early- and

mid-subjective night, respectively. This temporal expression pattern was also observed in *grk2*$^{+/-}$ and *grk2* cKO mice; however, in both strains the abundance of PER1 and PER2 nuclear staining at the single-cell level was augmented at the peak of their respective rhythms, resulting in a higher oscillatory amplitude. These effects on circadian amplitude were specific to PER1 and PER2, as they were not observed in the rhythms of BMAL1 expression in *grk2*-deficient SCN compared to wild-type controls.

Bioluminescent imaging of cultured SCN tissue explants was used to further examine the circadian pacemaking properties of the SCN in the absence of *grk2*. The *Per2:Luc* knock-in allele, which results in the production of the PERIOD2::LUCIFERASE fusion protein, was introduced into the *grk2*$^{+/+}$, *Vgat-cre::grk2*$^{flox/+}$ and *grk2* cKO backgrounds, and bioluminescent rhythms were subsequently monitored from cultured SCN explants for several weeks [27]. Compared with *grk2*$^{+/+}$ and *Vgat-cre::grk2*$^{flox/+}$ controls, *grk2* cKO SCN tissues exhibited a heightened amplitude of PER2::LUC oscillations, mirroring our immunohistochemical findings. *grk2* ablation does not appear to impact interneuronal coupling or communication within the SCN network, since the dampening rate of these oscillations was not markedly affected in *grk2*-deficient SCN. In contrast with the lengthened behavioural rhythms, the period of PER2::LUC rhythms was significantly shorter in *grk2* cKO SCN relative to its controls. Using murine embryonic fibroblasts, which exhibit circadian oscillations but do not couple to each other, PER2::LUC rhythms were also shortened upon silencing of *grk2* expression, suggesting that GRK2 has cell-autonomous effects on the period of molecular rhythms. From these results, we conclude that GRK2 regulates circadian amplitude and period length: genetic ablation of *grk2* shortens the intrinsic period of the SCN and at the same time augments SCN circadian amplitude. The discrepancy between the behavioural and molecular rhythms suggests that the period of behavioural rhythms is likely determined by interactions between the central pacemaker and peripheral oscillators elsewhere in the brain.

To shed light on potential mechanisms, we first turned our attention to the canonical function of GRK2 as a GPCR kinase. To this end, we used heterologous expression systems to probe the effects of wild-type and dominant negative GRK2 on the localization of various fluorescently-tagged GPCRs that are critical for SCN timing: VPAC2 (the receptor for VIP), V1b (for AVP), and PAC1 (for PACAP). Stimulation by the cognate ligand typically results in receptor internalization, which appears as cytoplasmic foci. For all three receptors tested, overexpression of wild-type GRK2 did not alter their localization at the plasma membrane under basal conditions, or their internalization upon ligand stimulation. However, in the presence of kinase-dead GRK2, ligand-induced VPAC2 and PAC1, and to a lesser extent V1b, internalization was suppressed. The in vitro effects of kinase-dead GRK2 on VPAC2 trafficking are reflected in the in vivo setting: VPAC2 expression at the protein, but not mRNA, level was significantly enhanced in the SCN of *grk2* cKO mice, suggesting that internalization-coupled degradation is reduced in the absence of *grk2*. However, VPAC2 upregulation appears to be compensated for by a reduction in VIP expression in *grk2*-deficient SCN. Given that VIP-VPAC2 signaling is crucial for intra-SCN coupling, compensation within the VIP-VPAC2 system could account for the absence of a dampening phenotype in the PER2::LUC experiments [28]. In light of these compensatory changes, it remains to be clarified which of the myriad circadian phenotypes arising from *grk2* ablation is attributable to the canonical actions of GRK2 on GPCR signaling.

To explain the effects of GRK2 on the abundance of PER1 and PER2 proteins, we examined the consequences of *grk2* ablation on *Period1/2* transcription. Within the SCN, the MAPK/ERK pathway is activated by light stimuli (through PACAP and glutamate signaling) and is upstream of period transcription [29]. Light-induced MAPK/ERK activity was markedly augmented in the SCN of *grk2* cKO mice in both the early and late subjective night, and may underlie their phase-shift phenotypes. Consistent with these findings, *grk2*$^{+/-}$ mice expressing the VENUS fluorescent protein under the control of the *mPer1* gene promoter showed greater *mPer1*-VENUS induction in response to early night light exposure and higher amplitude *mPer1*-VENUS rhythms [30]. *mPer1* transcript levels in the SCN were similarly affected in the absence of GRK2. Notably, *grk2* ablation did not alter *mPer2* mRNA levels in the SCN, suggesting that GRK2 regulates PER2 protein abundance in a manner independent of *mPer2* gene transcription. *mPer1*-luciferase assays in heterologous expression systems indicated that GRK2 regulates PACAP-induced *mPer1* transcription in a MAPK/ERK- and cAMP-dependent protein kinase (PKA)-dependent fashion. Collectively, these results suggest that GRK2 influences PER1 protein abundance, either partially or wholly, by a transcriptional mechanism that relies on MAPK/ERK and cAMP signaling. On the other hand, post-transcriptional mechanisms are likely responsible for GRK2's effects on PER2 protein levels. Potential mechanisms include changes in *Per2* translation or PER protein stability.

Acceleration of the circadian clock as a result of *grk2* ablation implies that the timing of the feedback inhibition is affected. One possibility is that GRK2 influences when and to which extent PER proteins accumulate in the nucleus. In

heterologous expression systems, overexpression of GRK2 suppressed nuclear accumulation of PER1 and PER2 and promoted their cytoplasmic retention. Conversely, a higher nuclear enrichment of PER2 and PER1 was observed in *grk2* siRNA-transfected cells and in dispersed SCN neuronal cultures derived from *grk2* cKO mice, respectively. These effects of GRK2 were specific to PER1 and PER2, and were not observed with the other murine core clock proteins. Collectively, our data reveal that GRK2 negatively regulates nuclear trafficking and accumulation of PER1 and PER2.

A number of other protein kinases, including casein kinase 1 delta/epsilon (CK1δ/ε), PKA and glycogen synthase kinase 3 beta (GSK3β), have been shown to directly or indirectly phosphorylate PER proteins and regulate their nuclear trafficking or protein turnover [10, 11]. To determine whether GRK2 is working upstream of one of these kinases to influence PER trafficking, we tested the effects of GRK2 overexpression or knockdown on PER1/2 localization in the presence of pharmacological inhibitors to various kinase pathways. Whereas GRK2-mediated PER1 cytoplasmic retention was sensitive to the CK1δ/ε inhibitor, PF-670462, and was unaffected by LiCl (inhibitor for GSK3β), U0126 (for MAPK/ERK) and H89 (for PKA), the nuclear-cytoplasmic distribution of PER2 in *grk2* siRNA-treated cells was affected by all of the inhibitors except for H89. In addition, U0126 and H89 altered the abundance of PER1 in the nucleus and cytoplasm, respectively, through potential effects on protein stability. Systemic administration of PF-670462 in wild-type and grk2 cKO mice abolished the phenotypic difference in nuclear PER1 and PER2 levels by substantially elevating their nuclear abundance in wild-type SCN. Given the dominant effects of CK1δ/ε inhibition on PER trafficking, and the fact that GRK2-dependent PER2 trafficking is sensitive to most of the pharmacological agents tested, it seems unlikely that GRK2 is merely functioning upstream of a particular kinase to regulate its direct action on PER proteins. Our data suggest an alternative scenario whereby multiple kinase pathways, including GRK2, impinge on PER1/2 and coordinately regulate their nuclear trafficking and accumulation.

Under this scenario, GRK2 may have a noncanonical function as a direct regulator of PER proteins. Co-immunoprecipitation experiments showed that GRK2 could physically associate with PER1 and PER2. On the other hand, GRK2 could not physically interact with CK1δ, and thus is unlikely to regulate CK1δ directly. Mass spectrometry-based phosphomapping of PER2 peptides derived from cells overexpressing wild-type or kinase-dead GRK2 revealed that GRK2 promoted PER2 phosphorylation on serine residue 545 in a manner that depended strictly on its kinase activity. This GRK2-mediated phosphorylation event was insensitive to CK1δ/ε inhibition. Collectively, our data suggest that GRK2 is a binding partner of PER1 and PER2, as well as a potential direct kinase of PER2. How the physical association with GRK2 and its phosphorylation on Ser545 affect the behaviour of PER2 remain to be determined. One or both of these events may be required to retain PER2 in the cytoplasm, thus delaying its entry into the nucleus. These events might also impact the stability of PER2 proteins.

Conclusions

Our study adds to a growing body of evidence that GRK2 is more than just a GPCR kinase (Fig. 1). Within the central circadian pacemaker of mammals, VIP and PACAP receptors are likely targets of GRK2: through receptor internalization, GRK2 circumscribes the activation of downstream signals, both in magnitude and in duration. The canonical interactions between GRK2 and such GPCRs as PAC1 receptors may underlie GRK2's effects on mPeriod1 gene transcription and thus its role in entrainment. However, we found that GRK2 also tangoes with PERIOD1/2 proteins, physically interacting with them and promoting the phosphorylation of PERIOD2. This novel function of GRK2 may mediate its effects on nuclear trafficking and accumulation of PERIOD proteins, and help to set the pace and amplitude of the circadian clock. Despite these tantalizing findings, we believe that we have only seen the tip of the GRK iceberg in terms of their roles in circadian timekeeping. Future studies will surely reveal more surprises for this multi-faceted family of protein kinases.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Abbreviations

AVP: arginine vasopressin; βTrCP: beta-transducin repeat

containing protein; CaMK: calmodulin-dependent protein kinase; CBP: CREB-binding protein; CK1: casein kinase 1; cKO: conditional knockout; CREB: cAMP-response element binding protein; DD: constant darkness; GPCR: G protein-coupled receptor; GRK: G protein-coupled receptor kinase; GRP: gastrin releasing peptide; GSK3: glycogen synthase kinase 3; LD: light-dark; LL: constant light; MAPK/ERK: mitogen-activated protein kinase/extracellular signal-regulated kinase; PACAP: pituitary adenylate cyclase-activating peptide; PKA: cAMP-dependent protein kinase; PP: protein phosphatase; PTM: post-translational modification; SCF: SKP1−Cullin1−F-box protein; SCN: suprachiasmatic nucleus; TTFL: transcription-translation feedback loop; UPS: ubiquitin-proteasome system; Vgat: vesicular GABA transporter; VIP: vasoactive intestinal peptide.

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