Efferent Projections of Prokineticin 2 Expressing Neurons in the Mouse Suprachiasmatic Nucleus

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Abstract

The suprachiasmatic nucleus (SCN) in the hypothalamus is the predominant circadian clock in mammals. To function as a pacemaker, the intrinsic timing signal from the SCN must be transmitted to different brain regions. Prokineticin 2 (PK2) is one of the candidate output molecules from the SCN. In this study, we investigated the efferent projections of PK2-expressing neurons in the SCN through a transgenic reporter approach. Using a bacterial artificial chromosome (BAC) transgenic mouse line, in which the enhanced green fluorescence protein (EGFP) reporter gene expression was driven by the PK2 promoter, we were able to obtain an efferent projections map from the EGFP-expressing neurons in the SCN. Our data revealed that EGFP-expressing neurons in the SCN, hence representing some of the PK2-expressing neurons, projected to many known SCN target areas, including the ventral lateral septum, medial preoptic area, subparaventricular zone, paraventricular nucleus, dorsomedial hypothalamic nucleus, lateral hypothalamic area and paraventricular thalamic nucleus. The efferent projections of PK2-expressing neurons supported the role of PK2 as an output molecule of the SCN.

Introduction

The primary mammalian circadian clock resides in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN drives the behavioral and physiological circadian rhythms, such as locomotor activity, sleep and wakefulness, feeding, energy metabolism and body temperature [1–3]. Dye tracing experiments have revealed that the primary efferent target areas of the SCN are quite limited and predominantly locate in the hypothalamus and the midline thalamus. Primary SCN target areas include lateral septum, bed nucleus of the stria terminalis, subparaventricular zone, paraventricular hypothalamic nucleus, dorsomedial hypothalamic nucleus as well as paraventricular thalamic nucleus [3–8]. Efferent projections of the SCN to its target sites are also inferred by the distributions of vasopressin (AVP) and vasoactive intestinal peptide (VIP) positive fibers, which largely overlaps with each other in all known SCN target sites within the hypothalamus [5,9]. The efferent projections of the mouse SCN correspond to what have been described in hamster and rat [4,6–8]. Both SCN subdivisions are believed to be capable of disseminating circadian information to the thalamus, hypothalamus and basal forebrain [9].

Transplantation of viable embryonic SCN tissues can partially restore the locomotor rhythm in SCN-lesion animals, and the rhythms restored by the transplants display the characteristics of the donor circadian pacemakers rather than those of the hosts [10–13]. Most interestingly, neuronal connectivity of the transplants was not established in these studies, suggesting that some humoral factors may be sufficient to transmit the circadian information from the SCN, at least for the locomotor rhythms. However, transplantation of embryonic SCN tissue does not restore the endocrine and other physiological rhythms, underscoring the importance of axonal connections between the SCN and its target sites for the regulation of many other circadian processes [14].

Prokineticin 2 (PK2) is a cysteine-rich secreted protein that exhibits high circadian rhythmic expression in the SCN, and its transcription is tightly controlled by components of the core molecular circadian oscillators [15–17]. Recently, genetic studies have revealed that PK2 regulates the circadian rhythms of locomotor activity, sleep and wakefulness, feeding and body temperature [18–19]. One receptor for PK2, prokineticin receptor 2 (PKR2), has been shown to be expressed in most primary target areas of the SCN by mRNA in situ hybridization [15,20], as well as ligand binding autoradiography [21]. In addition, targeted null mutation of the mouse PKR2 gene also disrupts the circadian rhythms, resulting in nearly identical phenotypes as the PK2 mutant mice [21–22]. One important perspective to comprehend the functions of PK2 in regulating the circadian system is to explore the characteristics of PK2-expressing neurons in the SCN and their connections in the context of neuronal circuitry. Unfortunately, no antibody against PK2, despite great efforts from multiple groups, was available for immunohistochemical study to date. In the current study, we obtained the projection map of a subset of PK2-expressing neurons in the SCN, utilizing a bacterial artificial chromosome transgenic mouse.

Results

Generation of PK2-EGFP transgenic mouse

To construct the transgenic mouse line, BAC clone RP23-12A18 was modified to insert an EGFP reporter cassette between the promoter and the first exon of PK2 gene (Figure 1A). For the
transgenic allele, transcription would be presumably driven by the PK2 promoter and stop after the polyadenylation site in the EGFP reporter cassette, resulting in EGFP expression without the overexpression of PK2. Indeed, we did not detect an increase in PK2 mRNA expression by in situ hybridization in the transgenic mice, compared with wild-type non-transgenic mice (data not shown).

Distribution of EGFP-expressing cells in the brain

We started by surveying the expression of EGFP reporter in the brains of 8–10 weeks old transgenic mice. Decent direct fluorescence in the cell soma could be seen in discrete areas of the adult brain, such as the olfactory bulb (OB), medial preoptic area (MPO) and SCN (data not shown). Either avidin-biotin based immunohistochemistry or indirect immunofluorescence staining significantly increased the EGFP signal, especially in the fibers. Since immunohistochemistry and immunofluorescence staining resulted in comparable signals, we used the latter to enable multiple signals detection.

As expected, many EGFP-immunostained (EGFP-ir) neurons were seen in the SCN (Figure 1G, see below for details). Besides that, copious EGFP-ir cells were seen in the granule layer (GL) and periglomerular layers (PGL) of the OB, with many cells exhibiting the morphologies of interneurons (Figure 1B). Quite a few EGFP-ir cells were also observed in subventricular layer of the lateral ventricle (LV), the rostral migration stream (RMS) and the subventricular layer of the lateral ventricle (LV). Most of these EGFP-ir cells looked like migrating neuroprogenitors, although a few appeared glia-like (Figure 1B, C and D). Many neurons in the dorsal tenia tecta (DTT), septohippocampal nucleus (SHi) and intermedial lateral septum (LSi) were also positive for EGFP (Figure 1D and E). The most intense signals for EGFP were seen in the preoptic area, including the anterodorsal preoptic nucleus (ADP) and MPO, while another group of EGFP-ir neurons were also observed in the horizontal limb of the diagonal band of Broca (HDB) (Figure 1F). There were also a handful of EGFP-ir neurons in the arcuate nucleus (Arc). No EGFP-ir cells were seen in the midbrain or the hindbrain (data not shown).

EGFP-immunostained fibers in most SCN target sites

Extensive EGFP-ir fibers were observed in most known SCN target areas in the septum, preoptic area, hypothalamus, thalamus and midbrain of the adult transgenic mouse brain (Figure 2 and Table 1). Dense EGFP-ir fibers could be seen coursing through the median preoptic area (MnPO), with many of them continued dorsally into the medial bed nucleus of the stria terminals (BSTM) and the ventral lateral septum (LSV) (Figure 2E and 2F). In the hypothalamus, the densest plexus of EGFP-ir fibers from the SCN began just dorsal and caudal to the nucleus, then vertically projected into the ipsilateral subparaventricular zone (SPa) and continued dorsally to a region ventral to the magnocellular part of
Figure 2. Distribution of EGFP-immunostained fibers in the SCN target areas and complementary expression of PKR2. A–D. Digoxigenin-labeled in situ hybridization showed the expression of PKR2. Arrows indicated PKR2-positive cells. DMH, dorsomedial hypothalamic nucleus; LH, lateral hypothalamic area; LHb, lateral habenular nucleus; LSD, dorsal lateral septum; LSV, ventral lateral septum; MnPO, median optical area; PVN, paraventricular nucleus; PVT, paraventricular thalamic nucleus. Scale bar = 200 μm. E–K. Immunofluorescence staining showed EGFP-ir fibers in E the median preoptic area (MnPO) and ventral lateral septum (LSV); F the subparaventricular zone (SPa) and paraventricular nucleus (PVN); G the lateral hypothalamic area (LH), dorsomedial hypothalamic nucleus (DMH) and arcuate nucleus (Arc); H the paraventricular thalamic nucleus (PVT); I the bed nucleus of the stria terminalis, medial (BSTM) and LSV; J the posterior hypothalamic area (PH) and medial supramammillary nucleus (SuMM) and K periaqueductal gray (PAG). Scale bar = 100 μm. L. A high magnification view of EGFP-ir cells and fibers inside the suprachiasmatic nucleus (SCN). Arrows indicated EGFP-ir fibers that extended from one nucleus into the contralateral nucleus. Scale bar = 20 μm. 3V, third ventricle.

The animals were sacrificed at ZT12. Cell nuclei were counter-stained in blue in E–L.
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the posterior paraventricular hypothalamic nucleus (PVN, Figure 2F). Inside the caudal part of SCN, a handful of EGFP-ir fibers could be seen crossing into the contralateral nucleus (Figure 2L). Posterior to the PVN, the dorsomedial hypothalamic nucleus (DMH) and lateral hypothalamic area (LH) received vast innervations of the EGFP-ir fibers. In contrast, there were few EGFP-ir fibers in the ventromedial hypothalamic nucleus (Figure 2G). A few EGFP-ir fibers were also observed in the Arc, posterior hypothalamic area (PH), lateral and medial supramammillary nucleus (SuMM, Figure 2J). In the thalamus, substantial EGFP-ir fibers could be seen extending dorsally and innervating the paraventricular thalamic nucleus (PVT) (Figure 2H). In the midbrain, intensive EGFP-ir fibers were observed throughout the length of the periaqueductal gray (PAG,

| Brain regions & Ventricles | Abbreviation | EGFP+ neurons | PK2 mRNA | EGFP+ fibers | PKR2 mRNA |
|----------------------------|--------------|---------------|-----------|--------------|-----------|
| Olfactory regions & Ventricles | GL | +++ | ++ | +++ | + |
| Granule layer | PGL | ++ | ++ | ++ | - |
| Periglomerular layer | OV | + | + | - | + |
| Olfactory ventricle | LOT | - | - | ++ | - |
| Lateral olfactory tract | Tu | - | - | ++ | - |
| Olfactory tubelel | RMS | + | + | - | ++ |
| Rostral migration stream | Subventricular zone of lateral ventricle | SVZ | + | + | + |
| Septum and Basal ganglia | LSD | - | - | - | + |
| Septum, Dorsal | LSI | +/+ | - | ++ | - |
| Septum, Intermediate | LSV | +/- | - | + | + |
| Septum, Ventral | SHi | ++ | - | ++ | - |
| Septohippocampal nucleus | ICJM | + | + | - | - |
| Major island of Calleja | DTT | ++ | - | ++ | - |
| Dorsal tenia tecta | PVA | - | - | + | +++ |
| Paraventricular thalamic nucleus, Anterior | PVT | - | - | +++ | ++ |
| Paraventricular thalamic nucleus | LHB | - | - | +/- | ++ |
| Lateral habenular nucleus | MPA | - | - | +++ | - |
| Medial preoptic area | LPA | - | - | ++ | - |
| Median preoptic area | MnPO | - | - | ++ | + |
| Vascular organ of the lamina terminalis | VOLT | - | - | ++ | - |
| Bed nucleus of the stria terminalis, Medial | BSTM | - | - | + | + |
| Medial preoptic nucleus | MPO | +++ | ++++ | ++++ | - |
| Anterodorsal preoptic nucleus | ADP | ++ | ++ | ++ | - |
| Horizontal limb of the diagonal band of Broca | HDB | + | - | + | - |
| Suprachiasmatic nucleus | SCN | +++ | +++ | ++ | +++ |
| Subparaventricular zone | SPa | - | - | +++ | + |
| Paraventricular nucleus | PVN | - | - | ++/+ | +/+ |
| Dorsomedial hypothalamic nucleus | DMH | - | - | +++ | - |
| Lateral hypothalamic area | LH | - | - | ++ | + |
| Arcuate nucleus | Arc | +/- | +/- | + | + |
| Posterior hypothalamic area | PH | - | - | + | - |
| Mammillary nucleus, Medial | MM | - | - | + | - |
| Mammillary nucleus, Lateral | ML | - | - | + | - |
| Supramammillary nucleus, Medial | SuMM | - | - | + | + |
| Supramammillary nucleus, Lateral | SuML | - | - | + | + |

| Midbrain | Abbreviation | EGFP+ neurons | PK2 mRNA | EGFP+ fibers |
|----------|--------------|---------------|-----------|--------------|
| Periaqueductal gray | PAG | - | - | ++ |
| Dorsal raphe nucleus | DR | - | - | + |

Table 1. Distribution of EGFP-immunoreactive neurons and projections in adult mouse brain.
Figure 2K), many of which probably extended into the dorsal raphe nucleus (DR, see Figure 3 and below for details).

It is important to note that many cells in these SCN target areas expressed $PKR_2$, a G-protein coupled receptor for PK2, as observed by in situ hybridization on adjacent sections (Figure 2A–D). For example, $PKR_2$ mRNA-expressing cells were seen in the MPO, LSV and LSD (Figure 2A), BSTM (data not shown), PVN, LH (Figure 2B), DMH (Figure 2C), Arc (data not shown), lateral habenular nucleus (LHb), PVT (Figure 2D) and DR (data not shown), suggesting that these areas were able to receive PK2 signaling from SCN.

Trajectory of the EGFP-ir axons could be evidently viewed in the sagittal plane (Figure 3). A great number of EGFP-ir neurons could be seen in the SCN, MPO and ADP. Dense plexus of EGFP-ir fibers were evident in the preoptic area, PVN, DMH and PVT. Particularly, a heavy bundle of EGFP-ir fibers could be seen curving posteriorly through the PVN and extensively innervating the DMH and posterior hypothalamic area. Many EGFP-ir fibers then exhibited an “S”-shaped trajectory along the boundary between thalamus and midbrain, and could be traced further into the DR (marked by arrows in Figure 3). There was a strong plexus of EGFP-ir fibers innervating the posterior PVT, coursing along the posterior boundary between thalamus and midbrain. A few of EGFP-ir fibers also curved along the anterior thalamus and extended into the PVT (Figure 3).

We used the retrograde axonal tracer Fluoro-Gold to confirm the origin of EGFP-ir fibers in the DMH, which receives inputs from other regions of the hypothalamus [23–24]. In four of twenty-four mice, Fluoro-Gold deposits were restricted primarily within the DMH (Figure 4). We focused our analysis on the distribution of retrogradely labeled neurons in the SCN and the preoptic area. Fluoro-Gold labeled neurons appeared to be widespread in the SCN. Remarkably, quite a few of the EGFP-ir neurons in the dorsomedial part of SCN were labeled by Fluoro-Gold (Figure 4G–I), indicating that these neurons projected into the DMH. In addition, significant numbers of neurons in the SPa and lateroanterior hypothalamic nucleus (LA) were also labeled by Fluoro-Gold (Figure 4D–F), consistent with known projection between these regions and the DMH. Retrogradely labeled neurons were also observed throughout the preoptic area (data not shown), in agreement with previous study on inputs to the DMH in the rat [23].

EGFP-immunostained neurons represented a subset of PK2 mRNA-expressing neurons in the SCN

The expression of $PK_2$ mRNA in the SCN exhibits profound circadian rhythm [15,25]. To explore whether the EGFP reporter imitated the daily rhythms of $PK_2$ mRNA, we characterized the regional and temporal distribution of EGFP-ir cells from the rostral to central (anterior and posterior portions) and caudal quadrants of the SCN during 24 hours (Figure 5). Overall, there were less EGFP-ir cells in the rostral and anterior central quadrants of SCN than in the posterior central and caudal quadrants (20±4 vs. 94±9 EGFP-positive cells on each 14 μm section at ZT12, p<0.001, non-parametric Kruskal-Wallis test,
three animals). During 24 hours, the number of EGFP-ir cells in the SCN showed a modest oscillation, declining to its nadir at ZT0 and escalating to its peak around ZT12 in both rostral and caudal halves of the SCN, with the latter showing greater oscillation amplitude (Figure 5. Effect of time: F(6,56) = 42.53, p < 0.001; Effect of position: F(3,56) = 219.45, p < 0.001; two-way ANOVA).

A closer look at the regional distribution of EGFP-ir neurons inside the SCN showed that the EGFP-ir neurons and processes were limited to the dorsomedial and lateral edges of the nucleus in the rostral and anterior-central compartments, creating the appearance of a shell. In the posterior central portion of the SCN, majority of the EGFP-ir cells were found in the medial and dorsomedial areas. While in the caudal quadrant, EGFP-ir cells dispersed throughout the SCN (Figure 5). As for the phenotype of the EGFP-ir neurons, about 60% of the EGFP-ir neurons in the SCN were also positive for vasopressin (AVP) at ZT12, as shown by double immunostaining with antibodies against AVP and EGFP (Figure 6A–D, EGFP+ cells: 606 ± 67; AVP+ cells: 2574 ± 190; EGFP+/AVP+ cells: 357 ± 32, three animals). On the contrary, no EGFP-ir cell was positive for vasoactive intestinal peptide (VIP) at the same condition (Figure 6E–H).

We also compared the expressions of endogenous PK2 mRNA and the EGFP reporter in the SCN (Figure 7). In situ hybridization against the EGFP mRNA showed a similar chronological expression pattern as the endogenous PK2 mRNA, reflecting the activity of the PK2 promoter used for the transgene approach (data not shown). As for the EGFP protein, a phase delay was observed when compared with PK2 mRNA. At ZT0, PK2 mRNA was expressed in only a few cells, then spread throughout the whole rostral-caudal extent of the SCN at ZT4 and diminished to the minimal level after ZT8. However, the peak of the EGFP protein expression was observed around ZT12 (Figure 7A and 7B, also see Figure 8).

At ZT4 in the SCN, only slightly more than 10% of the PK2 mRNA-expressing cells could be labeled by the EGFP reporter in the rostral and anterior central quadrants (13 ± 4 out of 124 ± 14 PK2+ cells were EGFP-ir on 14 μm section, three animals). In contrast, about 60% of PK2 mRNA-expressing cells were positive for EGFP signal in the posterior central and caudal SCN (29 ± 7 out
of 48±6 PK2+ cells were EGFP-ir on 14 μm section, three animals, also see Figure 3 for the preferential expression of EGFP reporter in the rostral SCN on parasagittal section). On the other hand, comparable (sixty) percentage of EGFP-ir cells in both the rostral (9±4 out of 13±4 EGFP-ir cells were PK2+ on 14 μm section, three animals) and caudal SCN (19±5 out of 29±4 EGFP-ir cells were PK2+ on 14 μm section, three animals) expressed PK2 mRNA at ZT4 (Figure 7). It is noteworthy that in the MPO, where the expression of PK2 mRNA doesn’t change over time, about 78% of the PK2 mRNA-expressing neurons were positive for EGFP reporter (47±8 out of 60±7 PK2+ neurons were EGFP-ir, three animals). Most importantly, all EGFP positive neurons in the MPO expressed the endogenous PK2 mRNA (data not shown).

Fluctuation of EGFP-ir fibers in many SCN target areas

Interestingly, we observed a wax and wane in the intensities of EGFP-ir fibers in many SCN target areas, following the same chronological pattern as the EGFP-ir neurons in the SCN (Figure 8). Side-by-side comparisons of coronal sections through the SPs, PVN, LH, PVT and PAG during 24 hours showed moderate oscillation of the amounts of EGFP-ir fibers in these areas, with the densest and strongest staining appeared around ZT12 and the lightest staining appeared around ZT0 (Figure 8). Density of the EGFP-ir fibers in the DMH, which also receive immense innervations from the MPO, did not show noticeable change during the 12L:12D cycle (data not shown).

Discussion

Previous studies have indicated that PK2 and its receptor PKR2 are important output components of the central circadian clock in the SCN [15,18–19,21]. The rhythmic expression of PK2 in the SCN and the complementary distribution of PKR2 in most primary SCN target areas [20–21] are consistent with the prospective role of PK2 as an output molecule to regulate multiple circadian rhythms in the rodent [15–16]. In this study, we confirmed that a subset of PK2-expressing neurons in the SCN projected to many primary target areas of the SCN. Given its secreted nature, these findings suggested that PK2 would be released at the terminals of efferent projections to regulate circadian-controlled processes.

To preserve most of the transcriptional units for PK2 gene expression, a BAC clone containing the PK2 gene and over 200 kb flanking sequences was used for the transgenic study. Overall, the distribution of EGFP-expressing cells in the brain of transgenic mouse matched the PK2 mRNA expression observed in previous in situ hybridization studies [17,20]. There were a few exceptions, however, such as many neurons in the dorsal tenia tecta (DTT) and septohippocampal nucleus (SHi) were positive for EGFP, but no evident PK2 mRNA expression was detected in these areas in the previous in situ hybridization study [20]. On the other hand, many cells in the islands of Calleja expressed PK2 mRNA, but only a few EGFP-ir cells were observed in the major island. This inconsistency might be caused by random insertion of the transgenic construct into the chromosome, a common unintentional outcome of the transgene approach. On the other hand, insertion of the EGFP reporter cassette after the PK2 promoter might nullify a potential enhancer in the first intron of PK2 gene. All these could contribute to the errant expression of the reporter gene in the transgenic mouse.

One distinctive characteristic of PK2 gene expression inside the SCN is its dramatic oscillation during the circadian cycle [15–17]. In
situ hybridization against the EGFP mRNA in the SCN showed a similar rhythmic expression pattern, reflecting the activity of PK2 promoter. Comparison of the dynamics of EGFP mRNA, PK2 mRNA and EGFP protein expression in the SCN showed that both EGFP and PK2 mRNA peaked at ZT4, although the peak of EGFP protein expression lagged 6–8 hours behind and peaked around ZT12, presumably due to some sort of delay in the protein translation process. On the other hand, when the EGFP and PK2 mRNA diminished to undetectable level at night, the EGFP protein persisted in the SCN, most likely owing to the long half-life (>24 hours) of the EGFP reporter used in this transgenic mouse [26].

EGFP reporter labeled a subset of PK2 mRNA-expressing neurons in the SCN

Using combined in situ hybridization against PK2 mRNA and immunostaining for the EGFP protein on the same frozen section, we found that as many as 80% of the PK2 mRNA-expressing neurons could be labeled by the EGFP reporter in the MPO, where the expression of PK2 mRNA was not oscillating during circadian cycle. However, only a subset of PK2 mRNA-expressing cells in the SCN was labeled by the EGFP reporter. In the posterior central and caudal quadrants of the SCN, the EGFP reporter was detected in more than 60% of PK2 mRNA-expressing neurons. While in the rostral and anterior central quadrants of the SCN, EGFP reporter only labeled about 10% of PK2 mRNA-expressing cells, mostly in the dorsomedial subregion. Taking into consideration of the phase delay between the PK2 mRNA and EGFP protein expression, the EGFP reporter would have labeled more PK2 mRNA-positive cells in this region. The transcription of PK2 gene has been supposed to be controlled by the binding of CLOCK/BMAL1 heterodimer to several E-box elements on the promoter of PK2 gene [15–16], which were preserved in the BAC cloned used in the transgene construct. However, the absence of the EGFP reporter expression in the majority of the PK2-expressing cells in the anterior part of the SCN suggested that the transcriptional regulation of PK2 gene in the anterior SCN neurons might be different from the posterior SCN counterparts. CLOCK/BMAL1 and the upstream promoter of PK2 gene would be sufficient for the activation of EGFP reporter in the posterior SCN. However, other transcription factors and a transcription enhancer, which most likely located in the first intron and was tampered by the insertion of the EGFP reporter cassette, would be essential for the authentic expression of PK2 gene in the anterior SCN.

A long-standing hypothesis assumes two separate, but mutually coupled, circadian oscillators that drive the onset and end of activity, and respond to dawn and dusk differentially [27–28]. Recent evidence suggest that differential oscillatory “evening” or “morning” machineries correspond to groups of neurons in the anterior or posterior divisions of the SCN [29–31]. As the EGFP reporter mainly represented the subset of PK2 mRNA-expressing cells in the central divisions of the SCN. It would be interesting to investigate whether these EGFP-positive neurons correlated to the “morning” cells. Further investigation on the characteristics of the EGFP-positive neurons would shed light on the role of these particular groups of neurons in the central circadian clock.

Figure 6. A subset of EGFP-ir cells in the SCN co-expressed vasopressin (AVP). A–C. Double immunostaining against EGFP and AVP in the SCN. Cells that were positive for both EGFP and AVP signals were marked by arrows. D. The localizations of EGFP- and AVP-positive neurons in the SCN was shown schematically. E–G. Double immunostaining of EGFP and vasoactive intestinal peptide (VIP) in the SCN. No neuron was positive for both EGFP and VIP signals. H. The localizations of EGFP- and VIP-positive neurons in the SCN were shown schematically. In all images, the boundary of SCN was indicated by the dotted line. The third ventricle was to the right in all images. The animals were sacrificed at ZT9. Scale bar = 20 μm. doi:10.1371/journal.pone.0007151.g006
Possible routes of PK2 in transmitting circadian information

PKR2, a G-protein coupled receptor for PK2, has been detected in major SCN target sites by in situ hybridization and ligand binding autoradiography [20–21]. In this study, we confirmed that the EGFP-labeled/PK2-expressing cells in the SCN projected to most SCN target areas, including the LSV, BSTM, MnPO, SPA, PVN, DMH, LH, PVT and PAG. Four different routes of projections for the EGFP-positive fibers could be traced out of the SCN. 1) The densest EGFP-ir fibers directed dorsally through the SPA and innervated the PVN; 2) Some EGFP-ir fibers turned caudally after leaving SCN and most likely went into DMH; 3) Rostrally, a few EGFP-ir fibers extended into the preoptic area, thereafter it may further extended into MnPO, BSTM and LSV; 4) Caudally, many EGFP-ir fibers appeared to target the posterior hypothalamus, especially the Arc. These findings suggested that PK2 might be axonally transported and released at the terminals, given its secreted nature, to regulate various circadian controlled processes by activating the receptor PKR2 expressed in these target sites. We observed little EGFP-ir fibers in some SCN target sites that were determined by dye-tracing and immunocytochemistry experiments, such as the anterior paraventricular thalamic nucleus and parataenial nucleus [4,6,9]. Considering that the EGFP reporter failed to label all PK2 mRNA-expressing cells in the SCN, we could not rule out the possibility of the PK2-expressing/EGFP-negative neurons in the SCN also projected to these sites.

During peak expression, the PK2-expressing cells are scattered in both the dorsomedial and ventrolateral SCN [see Figure 7A and [15,17,20]], which are two functionally and morphologically distinct sub-regions that are frequently recognized as vasopressin-(AVP) or vasoactive intestinal peptide-(VIP) expressing groups [13,32–34]. Immunohistochemical studies reveal AVP and VIP positive fibers originating from the SCN in all known SCN target sites within the hypothalamus, largely overlapping with each other [5,9]. In their double-labeled in situ hybridization study in the rat SCN, Masumoto et al [17] found nearly identical (about 50%) co-localization of PK2 mRNA with that of AVP or VIP. In this study, however, the vast majority of the EGFP-positive cells were located in the dorsomedial and caudal SCN, with most co-expressed with AVP and virtually none co-expressed with VIP. Even though approximately 60% EGFP-positive neurons in the SCN co-expressed AVP, there was a notable difference between the distribution of AVP-positive and EGFP-positive fibers in the target sites of SCN, particularly the limited presence of EGFP-positive fibers in the anterior paraventricular thalamic nucleus and ventromedial hypothalamic nucleus, suggesting that discrete groups of SCN neurons might have preferential projection targets.

Figure 7. The EGFP reporter labeled a subset of PK2 mRNA-expressing neurons in the SCN. A. On consecutive coronal sections of the SCN at ZT0, ZT4 and ZT8, fluorescence in situ hybridization for PK2 mRNA (in red color) and immunofluorescence staining against EGFP protein (in green color) revealed partial overlap between the PK2 mRNA and the EGFP reporter expressions. Most of the PK2 mRNA-expressing cells in the caudal part of the SCN were successfully labeled by the EGFP reporter, but the majority of PK2 mRNA-expressing cells in the rostral part of SCN did not express EGFP reporter. Scale bar = 50 μm. B. Quantification of PK2 mRNA-positive and EGFP protein-positive cells at different ZT time points. Error bars (SEM) were present in all data points, although some were too small to see.

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Interestingly, we observed a circadian variation in the intensities of EGFP-ir fibers in many of the SCN target sites, such as the PVN, LH and PVT, which bore a resemblance to the oscillation of EGFP expression in the SCN during 24 hours. Dye tracing experiments reveal that these areas also receive inputs from the MPO [24], another area which exhibits a strong co-expressing of PK2 mRNA and EGFP protein in the transgenic mouse. Nonetheless, the expression of EGFP reporter in MPO and other areas (such as OB, dorsal tenia tecta, septohippocampal nucleus, horizontal limb of the diagonal band of Broca and arcuate nucleus) did not change over the daily cycle, implying that the observed ebb and flow of EGFP-ir fibers in these areas were caused by changes of EGFP signals in the SCN. DMH, a hypothalamic nucleus involved in a variety of behavioral and physiological responses, has been considered as one of the major output targets of SCN [23]. Although there was little variation in the intensity of EGFP-ir fibers in the DMH, we confirmed that some of the EGFP-expressing neurons in the SCN indeed extended into DMH, using the retrograde tracer Fluoro-Gold.

Our study also demonstrated an innervation of the contralateral SCN, which has been reported in previous dye-tracing experiment in hamster [6]. In rat, vasoactive intestinal polypeptide (VIP) containing fibers could be observed to traverse the optic chiasm in immunostaining studies [35]. It has been suggested that the reciprocal innervation of the bilateral SCN serves to couple the two distinct circadian oscillators, which seems to correspond to the left and right sides of the bilaterally paired SCN [36–38]. PKR2 mRNA-expressing neurons are clustered in the dorsolateral region of the SCN [17], which has important roles to relay or integrate the phase-resetting information to autonomously oscillating cells [39–40]. It is plausible that PK2-PKR2 system might play some role in integrating the circadian phases of the two paired SCN.

In summary, this study showed that PK2-expressing cells in the SCN projected into many known SCN target sites, indicating that PK2 could reach these sites through axonal transportation. Further studies are warranted to determine the role of distinct groups of PK2-expressing cells in the SCN and how PK2 is released at the terminals and transmits the circadian information of the central clock.
Materials and Methods

Transgenic animal
A PK2-EGFP transgenic mouse was generated by the GENSAT project at Rockefeller University [41]. Briefly, the bacterial artificial chromosome (BAC) clone RP23-12A18 was genetically modified so that the enhanced green fluorescence protein (EGFP) gene followed by a polyadenylation signal was inserted after the promoter of PK2 gene (Figure 1A). The 250 kb BAC clone contained the entire transcriptional unit for PK2 gene, with 127 kb upstream and 110 kb downstream sequences. Cryopreserved embryos (011832-UCD-Embryos) of PK2-EGFP transgenic mice were obtained from the Mutant Mouse Regional Resource Centers (MMRRC, University of California at Davis) and recovered by the Transgenic Mouse Facility at University of California, Irvine. Presence of the transgene was determined in mouse tail genomic DNA by PCR, using primer1 (5'-CGTACGCGTGCAGTGCTTCAGC-3') and primer2 (5'-CGGCAGGTCGAGCTGCACGCTGCCGTCCTC-3'). Homozygous transgenic mice were bred from hemizygous and used in further experiments. PK2 embryonic stem cells were obtained from the Transgenic Mouse Facility at Rockefeller University [41]. These ES cells contained the entire transcriptional unit for PK2 gene (Figure 1A). The 250 kb BAC clone was modified so that the enhanced green fluorescence protein (EGFP) gene was expressed under the control of a Mcp promoter. The Mcp promoter contains the transactivation domain of the neuron-specific enolase promoter, which is expressed in neurons during development.

Perfusion and immunofluorescence staining
Eighteen mice of 8–10 weeks age, three for each time point, were sacrificed at zeitgeber time (ZT) 0, 4, 8, 12, 16 and 20. Animals sacrificed in the dark were deeply anesthetized with pentobarbital (150 mg/kg) and perfused intracardially between 6:30PM to 7:30PM as described above. The brain was removed, post-fixed for seven days, animal was deeply anesthetized with pentobarbital (150 mg/kg) and perfused intracardially between 6:30PM to 7:30PM as described above. The brain was removed, post-fixed overnight at 4°C and cryosectioned. Forty-micron sections were collected. For those animals with the Fluoro-Gold deposits restricted primarily within the borders of the DMH, immunodetection of EGFP-expressing cells within the SCN was performed using rabbit anti-GFP antibody. Fluoro-Gold labeled neurons were detected by direct fluorescence.

Image analysis
For immunofluorescence staining and Fluoro-Gold tracing, multichannel fluorescence images of the brain sections were captured using a CCD camera attached to a Carl Zeiss Axiostar microscope (Axiostar 200M) and exported as TIFF image files using Axiostar LE (Carl Zeiss, Germany). For DIG-labeled in situ hybridization, images were captured using a SPOT camera system (Diagnostic Instruments Inc., Sterling Heights, MI) attached to a Carl Zeiss Axiostarplus 2 Plus light microscope. Adobe Photoshop was used to adjust the brightness and contrast of images, so that the background of all images appeared similar.

Cell counts
Only cells with positive signals stronger than the background were tallied for quantification. Cell counts were performed by counting all cells within the boundaries of the SCN in each section, and total cell number in the whole SCN were estimated using the stereological method as described [9]. Statistical analysis was carried out with GraphPad Prism software.

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Author Contributions
Conceived and designed the experiments: CZ QYZ. Performed the experiments: CZ KKT. Analyzed the data: CZ KKT. Wrote the paper: CZ QYZ.
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