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Research report

Differential expression of protein kinase C βI (PKCβI) but not PKCα and PKCβII in the suprachiasmatic nucleus of selected house mouse lines, and the relationship to arginine-vasopressin

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Abstract

The functional significance of the suprachiasmatic nucleus (SCN) in circadian rhythm control of mammals has been well documented. The role of protein phosphorylation mediated by protein kinase C (PKC), however, is not well known. We report the immunocytochemical localization of three Ca^{2+}-dependent PKC isoforms (α, βI, βII) within the SCN of selected house mouse lines that differ in behavioral circadian rhythm parameters. Optical density measurements revealed that the adult mice selected for low levels of nest-building behavior (small nest-builders) had more than threefold higher PKCβI immunostaining in the SCN than the mice selected for high levels of nest-building behavior (big nest-builders). A similar twofold difference between the adult small and big nest-builders was observed for the number of PKCβI-containing cells in the SCN. The non-selected control lines were intermediate. Ten-day-old pups revealed similar differences in PKCβI immunostaining in the SCN between the small and big nest-builders. PKCα and PKCβII immunostaining in the SCN was not different among the lines. PKCβI immunostaining was not different among the selected lines in the lateroanterior hypothalamic nucleus (LA) and the cornu ammonis field 1 (CA1) of the dorsal hippocampus and confirms the specificity of the difference in PKCβI immunostaining in the SCN among the selected lines. The significance of these findings is discussed in the context of differences among the lines in arginine-vasopressin (AVP) and light-induced Fos expression in the SCN, behavioral phase-delay responses to 15-min light pulses in constant darkness, and measures of the strength of the circadian activity rhythm expressed. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Neural basis of behavior

Topic: Biological rhythms and sleep

Keywords: Suprachiasmatic; Protein kinase C; Mouse; Circadian; Vasopressin; Signal transduction; Fos

1. Introduction

The functional significance of the suprachiasmatic nucleus (SCN) in circadian rhythm control of mammals has been well-documented [32]. Cycles of protein phosphorylation play key roles in the Drosophila circadian clock [23] and are also very important in the function of the SCN, e.g., a mutation in casein kinase I epsilon is responsible for the tau-mutant hamster phenotype [18]. The characterization of a molecular basis for a 24-h cycling mechanism in mammals is well on its way, including how the molecular circadian clock regulates arginine-vasopressin (AVP) expression in the SCN [15]. This work and that of others also reveal the importance of protein phosphorylation, intimately linked to ion exchange (Ca^{2+}) and second messenger activation [10], in the function of the mammalian circadian clock [22], including protein kinase C (PKC) [2,7,11,12,14,16,20,30].

In the mouse SCN, PKCα, one of four identified Ca^{2+}-dependent PKC isoforms (α, βI, βII, γ), is present in all AVP-positive and many AVP-negative neurons. In addition, double-labeling immunocytochemistry revealed that AVP
boutons terminate on PKCα neurons in the SCN of these mice [30]. AVP neurons in the SCN receive synaptic connections from other AVP neurons in the SCN, which results in an interconnected network of these neurons in the SCN [9,29]. The distribution of PKCα within the mouse SCN suggests that it may be involved in signaling between AVP neurons as well as between AVP-positive and AVP-negative neurons [7,30]. AVP stimulates SCN neurons by activation of the V1-like class of receptors [17]. This receptor activation leads to phosphoinositol turnover and the subsequent release of intracellular Ca²⁺ from endoplasmic reticulum stores, which is necessary for the activation of PKC isoforms [24]. In addition, PKC also modulates neurotransmitter release presynaptically [19] and therefore PKCα might directly influence AVP release from PKCα-positive AVP neurons in the SCN. Intracellular Ca²⁺ has been shown to play a role in the SCN but whether intracellular Ca²⁺ changes in SCN neurons [27,28] lead to the activation of Ca²⁺-dependent PKC isoforms in the SCN remains to be elucidated. A functional relationship between AVP and PKCα in the SCN has been suggested for the common vole (Microtus arvalis) because the numbers of AVP- and PKCα-immunoreactive neurons decrease concurrently from zeitgeber time 0 (ZT 0) to ZT 6 [14].

Bidirectional selection for thermoregulatory nest-building behavior resulted in a correlated response for the number of AVP neurons in the SCN [8]. The differences in the number of AVP neurons in the SCN among the selected lines correlate with several circadian rhythm parameters of wheel-running activity. Larger numbers of AVP neurons in the SCN are correlated with higher activity levels in a free-running period in DD by immersion fixation for 24 h as described previously [14]. Immersion fixation was chosen since transcardial perfusion at this age is difficult. The brains were cut into 20-μm coronal sections on a cryostat microtome. Three sets of sections per brain were collected so that each animal could be immunocytochemically stained for the three PKC isoforms.

For double-labeling immunofluorescence for AVP and PKCβI, four adult male control mice, 282–286 days of age, were anesthetized 4–5 h after lights on (ZT 4–5; in a 12:12 LD cycle) and their brains processed as described above.

For PKCα, PKCβI and PKCβII immunostaining, eighteen adult male mice, 50–100 days of age, (six big nest-builders, six small nest-builders, and six non-selected control mice) were anesthetized 3–8 h after lights on (ZT 3–8; in a 16:8 LD cycle) and the animals were transcardially perfused as described previously [30]. The big and small nest-builders and control mice were killed alternately to avoid a bias in the time the brain was obtained. The brains were cut into 25-μm coronal sections on a cryostat microtome. Three sets of sections per brain were collected.

2. Materials and methods

2.1. Animals

*Mus domesticus* lines, bidirectionally selected for nest-building behavior for over 50 generations were used for this study [4–6]. The mice were raised on wood shavings in polypropylene cages (27x17x12 cm) under a 16:8 or 12:12 LD cycle at 22±1°C. The young were weaned at 21 days of age and housed with like-sexed littermates until the start of the experiment. Food and water were available ad libitum.

For PKCα, PKCβI and PKCβII immunostaining, eighteen adult male mice, 50–100 days of age, (six big nest-builders, six small nest-builders, and six non-selected control mice) were anesthetized 3–8 h after lights on (ZT 3–8; in a 16:8 LD cycle) and the animals were transcardially perfused as described previously [30]. The big and small nest-builders and control mice were killed alternately to avoid a bias in the time the brain was obtained. The brains were cut into 20-μm coronal sections on a cryostat microtome. Three sets of sections per brain were collected so that each animal could be immunocytochemically stained for the three PKC isoforms.

For double-labeling immunofluorescence for AVP and PKCβI, four adult male control mice, 282–286 days of age, were anesthetized 4–5 h after lights on (ZT 4–5; in a 12:12 LD cycle) and their brains processed as described above.

2.2. Immunocytochemistry

2.2.1. Single labeling

Free-floating immunocytochemical methods as described by Van der Zee and Bult [30] were used to characterize the immunocytochemical distribution of PKCα, PKCβI, and PKCβII in the SCN. In short: after washes and a blocking step, the sections were incubated overnight at 4°C with polyclonal rabbit IgG anti-PKCα (1:100 or 2 μg/ml), anti-PKCβI (1:100 or 2 μg/ml), or anti-PKCβII (1:100 or 2 μg/ml) (C-20, C-16, and C-18, respectively; Santa Cruz Biotechnology; for discussion of the specificity of these antibodies see Refs. [7,30]) in 0.01 M phosphate-buffered saline (PBS, pH 7.4). Then the brain sections were rinsed and incubated for 2 h at room temperature with biotinylated goat anti-rabbit IgG (F(ab′)2 fraction, 1:200; Amersham) in PBS containing 5% normal goat serum (NGS). After rinses in PBS, the tissue was incubated with
Streptavidin–HRP (1:200; Amersham) in PBS for 2 h at room temperature. The sections were again rinsed in PBS and Tris buffer, and reacted under visual guidance with diaminobenzidine (DAB; 30 mg DAB in 100 ml Tris buffer, pH 7.4) and 0.01% H₂O₂. Finally, the sections were mounted and coverslipped for light microscopic analysis.

2.2.2. Double-labeling immunofluorescence for PKCβI and AVP

Free-floating double-labeling immunofluorescent methods were adapted from Van der Zee and Bult [30] and used to quantify the co-localization of PKCβI and AVP within the same neurons in the SCN of control mice. In short: after washes in PBS, brain sections were blocked in 5% NGS in PBS. Subsequently, the sections were incubated for 48 h at 4°C with polyclonal rabbit IgG anti-PKCβI (1:500 or 0.4 μg/ml; C-16, Santa Cruz Biotechnology) and mouse monoclonal IgG anti-AVP (1:100; PS41, kindly provided by Dr. H. Gainer) in 3% NGS in PBS. Then the brain sections were rinsed in PBS and incubated for 2 h at room temperature with biotinylated goat anti-rabbit IgG (1:200; Vector) and horse anti-mouse IgG conjugated to Texas Red (1:100; Vector) in 3% NGS in PBS. After rinses in PBS, the tissue was incubated with Avidin-D conjugated to fluorescein (1:167; Vector) in PBS for 1.5 h at room temperature. The sections were again rinsed in PBS, mounted, and coverslipped in Gel/Mount (Biomedex). Fluorescent microscopic analysis was performed on a fully motorized Zeiss Axioplan 2 Imaging fluorescent microscope equipped with an AxioCam digital camera and AxioVision 2.0 software.

2.3. Relative optical density measurements

The level of PKCα, PKCβI, and PKCβII immunostaining was assessed with the Quantimet 600 analysis system (Leica) according to the methods of Van der Zee et al. [31]. The optical density (OD) of the staining was measured simultaneously in the left and right side of the SCN in two coronal sections in the middle of the SCN with a 10× objective lens. The OD, therefore, reflects the average of four separate SCN sections. Visual inspection of anterior and posterior sections of the SCN revealed similar levels of staining compared to the middle sections of the SCN in the same individual. Therefore, a middle section of the SCN is representative of the levels of staining in the entire SCN. The OD of PKCβI immunostaining was also assessed in two coronal sections in the left and right lateroanterior hypothalamic nucleus (LA) and the cornu ammonis field I (CA1) of the dorsal hippocampus. The OD of each individual reflects the average of four separate measurements for both brain regions. The LA was measured at the level of the SCN and the CA1 was measured directly posterior to the SCN in the same tissue that was used for the analysis of immunostaining in the SCN. The LA was clearly delineated by PKCβI immunostaining and the OD of PKCβI immunostaining was measured in the entire LA in each section. In the CA1 only the pyramidal cell layer (stratum pyramidale), which revealed the highest level of PKCβI immunostaining, was measured according to the methods of Van der Zee et al. [31]. PKCα and PKCβII staining were measured with a yellow filter and PKCβI was measured with a green filter. Different filters were used to optimize the staining density distribution between full saturation and no signal at all. To be able to compare the level of staining among the three PKC isoforms at the level of the yellow filter, a correction factor was determined for the PKCβI staining of 21.3, i.e., the OD measurements with the green filter were 21.3 times as high as the yellow filter.

The OD was corrected for different levels of background staining between individuals by calculating a standardized relative optical density: \( \left( \frac{\text{OD} - \text{OD}_{\text{background}}}{\text{OD}_{\text{background}}} \right) \). In adults for PKCα, the OD_{\text{background}} was measured in an area directly lateral to the SCN that was devoid of PKCα-specific staining. In adults for PKCβI and PKCβII, the optic chiasm was used for the OD_{\text{background}} measurement because the hypothalamic area contained too much PKCβI- and PKCβII-specific staining. The levels of background staining in the hypothalamus and the optic chiasm were identical for PKCβI and PKCβII staining (OD_{\text{background}}=0.25±0.03). For the PKCβI staining the OD_{\text{background}} in the optic chiasm was 0.14±0.03. This lower OD_{\text{background}} may have increased the relative optical density measurement of PKCβI staining compared to PKCα and PKCβII staining. However, this had no effect on the general findings of this study since the level of PKCβII staining was well below that of the other two isoforms (see Section 3). One small nest-builder had to be excluded from the analysis because of poor fixation of the brain tissue. For the PKCβI immunostaining in the 10-day-old pups, the paraventricular nucleus of the hypothalamus (PVN) was used to determine the OD_{\text{background}} because it was devoid of PKCβI-specific staining. The optic chiasm or the area directly lateral to the SCN could not be used because these areas contained too much PKCβI-specific staining. The OD_{\text{background}} was 0.39±0.02 and 0.41±0.01 for the small and big nest-builders, respectively, and were not significantly different (\( t_{12}=1.012, \text{n.s.} \)).

2.4. Cell counts

PKCβI-immunopositive and AVP-immunopositive neurons were counted in two sections containing the medial SCN. PKCβI-immunopositive neurons were only counted when the nucleus was in the plane of the section and the PKCβI staining was observed as a rim of immunodeposits along the cell membrane. For the 10-day-old pups, AVP-immunopositive neurons were only counted when the nucleus was in the plane of the section and AVP staining in the cytoplasm was present surrounding the nucleus. Summed cell counts were used as the individual values for statistical analysis.
For the evaluation of PKCβI and AVP co-localization, the percentage of AVP neurons containing PKCβI immunofluorescence was determined in an anterior, middle, and posterior section through the SCN. The left and right SCN within each section were analyzed simultaneously.

2.5. Reanalysis of wheel-running activity data from Amy et al. [1] and Bult et al. [8]

The reanalysis of wheel-running activity data was performed to enable a more detailed correlative study of the current neurochemical analysis with the strength of the circadian organization of locomotor behavior. The mice were raised on wood shavings in polypropylene cages (27×17×12 cm) under a 12:12 light–dark (LD) cycle at 22±1°C [1]. An additional group of animals, that was used to determine the fragmentation of the activity pattern, was maintained under a 16:8 LD cycle [8]. At approximately 50–100 days of age, two separate groups of 24 male mice (eight high- and eight low-selected, and eight non-selected control mice; the data from one group were reanalyzed from Ref. [1]) were placed individually in polypropylene cages (24×35×21 cm) equipped with a 27-cm running wheel, and wheel running activity in 5-min intervals was recorded continuously by the VitalView computerized data collection system (Mini Mitter Co, Bend, OR). For the determination of the fragmentation of the rhythm, three additional groups (16 animals per group, divided over the three selected lines) were used and these represent a reanalysis of data from Ref. [1]. Food and water were available ad libitum. Wheel running activity was monitored for 2 weeks under a 16:8 LD cycle followed by DD.

Within the first two weeks of DD, using 7–10 days of continuous recordings, the fragmentation and amplitude of the activity rhythm, and relative tau peak height were measured. These characteristics are thought to represent measures of the strength of the circadian rhythm expressed. The free-running period in DD (τDD) was calculated with the TAU software program (Mini Mitter Co, Bend, OR). Actograms were subsequently double plotted at a τDD day length. The amount of fragmentation was determined by measuring the average number of activity bouts per circadian day during the 7–10 day period, in which a larger amount of fragmentation is considered a characteristic of weaker circadian control. The activity level had to be above 15% of maximum activity during any 10-min interval and activity bouts had to be apart by at least 20 min to be counted individually. The mean maximum activity level per hourly interval was used as the amplitude of the rhythm when the activity was plotted as number of wheel revolutions per hour over the circadian day, in which a lower amplitude is considered a characteristic of weaker circadian control. An increased tau peak height is assumed to indicate a stronger circadian pacemaker. Since the periodogram of the TAU program automatically scales down with increased tau peak height, the relative tau peak height had to be calculated to be able to appropriately compare among the selected lines. The relative peak height was calculated by dividing the distance between the top of the peak and the 5% significance diagonal by the distance between the x-axis and the 5% significance diagonal. If the 5% straight diagonal line is a significance criterion based upon the chi-square statistic [26]. Four animals had to be excluded from the analyses because they did not express high enough activity levels to determine their rhythms accurately.

2.6. Statistical evaluation

In order to test whether the selected lines (adults) were significantly different, we analyzed differences in relative optical density and the number of immunopositive cells of PKCα, PKCβI, and PKCβII immunostaining in the SCN and PKCβI immunostaining in LA and CA1 using the SAS General Linear Models (GLM) procedure one-way analysis of variance (ANOVA) for effect of Selected Line (big nest-builder, control, small nest-builder). Differences in the relative optical density within each line were tested using a SAS GLM one-way ANOVA for effect of PKC Isoform (α, βI, βII). When significant Selected Line or PKC Isoform effects were found, pairwise comparisons were made using the Tukey Studentized Range test (Student t-test equivalent) with a significance level of P<0.05. For the 10-day-old pups, differences in relative optical density and the number of immunopositive cells of PKCβI and AVP immunostaining in the SCN were statistically evaluated with a Student t-test. For the evaluation of PKCβI and AVP co-localization, the percentage of AVP neurons containing PKCβI immunofluorescence was arcsine transformed and analyzed with a one-way ANOVA for effect of Section (anterior, middle, posterior).

Differences among the selected lines in the fragmentation and amplitude of the activity rhythm, and relative tau peak height were evaluated using the SAS GLM procedure, for effects of Selected Line (big nest-builder, control, small nest-builder) and Group (i.e. first group of 24 animals, second group of 24 animals, and for the fragmentation of the activity rhythm: three additional groups of 16 animals) nested within Selected Line. The Selected Line and Group Variances were pooled if the significance level of the Group effect was P>0.20. If the Group effect was significant, the Group variance was used as the error term for the Selected Line effect. All results are expressed as mean±standard error.

3. Results

The immunocytochemical distribution of PKCα, PKCβI, and PKCβII in the SCN of non-selected adult mice was very similar to that reported previously [30]. PKC im-
munostaining was primarily associated with the neuronal cell membrane, while the nucleus remained immunonegative. PKCα and PKCβI were distributed throughout most of the SCN with more intense staining in the dorsomedial and ventrolateral subdivisions of the SCN (Fig. 1). These subdivisions also contain AVP [8,30]. PKCβII staining was more sparsely distributed and immunopositive cells were most abundant at the dorsomedial and lateral edges of the SCN (Fig. 1c).

The quantitative (relative optical density) measurements of PKCα, PKCβI, and PKCβII correspond to our earlier semi-quantification assessment of these PKC isoforms in the non-selected mouse SCN [30]. The adult small nest-builders contained more than three times the amount of PKCβI staining in the SCN compared to the adult big nest-builders ($F_{2,14}=5.49$, $P<0.018$). The adult control mice were intermediate in PKCβI staining (Figs. 1 and 2). The relative optical densities of PKCα and PKCβII immunocytochemical staining in the SCN were not different among the selected lines (Fig. 2; $F_{2,14}=0.00$, n.s.; $F_{2,14}=0.49$, n.s., respectively). Therefore, PKCα and PKCβII staining serve as controls within the SCN for the PKCβI staining differences among the lines, especially since all three PKC isoforms were stained in brain tissue from the same animals. In general, PKCα was the most highly expressed Ca$^{2+}$-dependent PKC isoform in the mouse SCN, followed by PKCβI. PKCβII had the lowest expression level. However, in the small nest-builders, the PKCβI staining level was equivalent to that of PKCα (Figs. 1 and 2).

In addition to a higher level of PKCβI immunostaining in the SCN of the adult small nest-builders, the neuronal density of PKCβI-positive cells was also higher, with intermediate values for the control mice (Fig. 3).

In 10-day-old pups, the relative optical density of PKCβI immunostaining and the number of PKCβI-positive cells in the SCN were significantly higher in the small nest-builders compared to the big nest-builders (Figs. 3 and 4; $t_{12}=3.775$, $P<0.005$). These results obtained at ZT 3–4 confirm the findings in the adult male mice. The brains of the adults were obtained over a longer time interval during the light period, and possible circadian variation in PKCβI expression might have increased variability among individuals. However, among selected line differences in PKCβI immunostaining were apparently robust in the adult mice and were replicated in the 10-day-

![Fig. 1. Representative photomicrographs of PKCα (a), PKCβI (b,d,e), and PKCβII (c) immunoreactivity in the SCN of adult small nest-builders (d), control mice (a,b,c), and big nest-builders (e). Scale bar=70 μm.](image-url)
old pups. Interestingly, the number of AVP-positive neurons in the SCN was not yet different at 10 days of age (315.4±30.6 (n=7) and 300.7±27.2 (n=7) for the small and big nest-builders, respectively, \( t_{12}=0.949, \text{n.s.} \)). In adults, the small nest-builders have a 1.5-fold larger number of AVP neurons in the SCN than the big nest-builders and control mice (Ref. [8]; E.A. Van der Zee, D.R. Van der Veen, K. Jansen, M.P. Gerkema, A. Bult, unpublished data).

Fig. 2. Relative optical densities of PKCa, PKCbI, and PKCbII immunoreactivity in the SCN of big nest-builders, control mice, and small nest-builders. Adults: The small nest-builders contained more than three times the amount of PKCbI staining in the SCN compared to the big nest-builders \( (F_{5,14}=5.49, *P<0.018) \). Within each line (big nest-builder \( (F_{5,15}=5.88, P<0.013) \), control \( (F_{5,15}=5.01, P<0.022) \), small nest-builder \( (F_{5,12}=3.45, P<0.066, \text{n.s.}) \)), different letters indicate significant differences at \( P<0.05 \) of pairwise comparisons using the Tukey Studentized Range test. Ten-day-old pups: \( **t_{12}=3.775, P<0.005 \).

Fig. 3. Number of PKCbI-positive cells in two medial sections in the SCN of big nest-builders, control mice, and small nest-builders. Different letters indicate significant differences at \( *P<0.05 \) of pairwise comparisons using the Tukey Studentized Range test (adults: \( F_{5,15}=18.73, P<0.0001 \); 10-day-old pups: \( t_{12}=5.23, P<0.0005 \)). No data were available for control pups.

Fig. 4. Representative photomicrographs of PKCbI immunoreactivity in the SCN of a 10-day-old big nest-builder (a) and a 10-day-old small nest-builder (b). Scale bar=70 \( \mu \)m.
Double-labeling immunofluorescence for PKCβI and AVP revealed that of 1495 AVP neurons studied 1424 (95.0±1.2%; n=4) also expressed PKCβI. About 60% of the PKCβI cells did not contain AVP (Fig. 5). The percentage of AVP neurons containing PKCβI was similar from anterior to posterior in the SCN (F2,9=2.18, n.s.). In addition, apparent vasopressinergic boutons were observed in close proximity to PKCβI immunopositive cells (data not shown).

Reanalysis of wheel-running activity data of Refs. [1,8], necessary for the functional interpretation of the immunocytochemical results, revealed weaker organization in circadian expression in the big nest-building compared to the small nest-builders, exemplified by increased fragmentation, decreased relative tau peak height, and decreased amplitude of the activity rhythm in the big nest-builders. The control mice were intermediate for these circadian characteristics (Fig. 6).

Fig. 5. Representative digital photomicrographs of fluorescent PKCβI (a) and AVP immunostaining (b) in the same SCN tissue section. The arrows highlight several neurons double-labeled for AVP and PKCβI. The arrowheads highlight PKCβI cells that do not contain AVP. 3V, third ventricle; OC, optic chiasm. Scale bar=90 μm.
4. Discussion

The more than threefold higher level of PKCβI expression in the SCN of the small nest-builders compared to the big nest-builders, with the control mice having intermediate values, was unexpected but may be related to the strength of circadian rhythms expressed. These results reveal an inverse relationship between the amount of PKCβI in the SCN and the phase-delay response and number of Fos nuclei expressed in the SCN after a 15-min light pulse in the subjective night, e.g., at CT 16 in DD (Table 1) [1,3]. Assuming that a more strongly organized circadian pacemaker is less likely to be perturbed by environmental stimuli, the smaller phase-delay responses to a 15-min light pulse of the small nest-builders possibly indicates a more strongly organized pacemaker compared to big nest-builders that show 2–3 times larger phase-delay responses to light. This difference in strength of the rhythms expressed correlates positively with the level of wheel-running activity and amplitude of the activity rhythm (Table 1). However, the control mice are similar for AVP cell numbers in the SCN compared to big nest-builders, but different from them for PKCβI immunoreactivity in the SCN, light-induced phase-delay responses, fragmentation of the activity rhythm, and relative tau peak height (Table 1). These apparent discrepancies may reflect an interaction between the AVP and PKCα/PKCβI neuronal populations. If AVP signaling to other AVP neurons as well as neurons lacking AVP could employ either PKCα or PKCβI as second messengers, the effects might be different depending on the activated PKC isoform. This is a realistic possibility since most AVP neurons in the mouse SCN contain PKCα [30] and PKCβI (this study). In addition, AVP-negative SCN cells expressing PKCα [30] and PKCβI (this study) also appear to receive vasopres- sinergic innervation.

Alternatively, the differences observed among the selected lines in the strength of circadian activity rhythms may not be due to differences in pacemaker function but may reflect different responses of target areas to similar SCN signals. Supporting evidence for this hypothesis was found in a comparison of circadian expression patterns of Fos immunoreactivity in the SCN and several hypothalamic regions in the nocturnal rat and the diurnal Arvicanthis niloticus. The results of this study indicate that nocturnality and diurnality may partially depend on different responses of hypothalamic targets to SCN signals [21]. In addition, different retinal input may also explain the differences observed in light-induced phase shifts and Fos expression in the SCN among the selected lines.

PKCβI might also have a direct inhibitory role on light-induced responses, which may be unrelated to AVP. Evidence suggests that PKC inhibits ionotropic glutamate receptor mediated transduction of light information from the retina to the SCN through a metabotropic glutamate receptor pathway [12]. We hypothesize that PKCβI may be
responsible for this inhibitory role in glutamate mediated signaling since the small nest-builder with the highest levels of PKCβI in the SCN showed the smallest light-induced phase shifts. Other evidence suggests that PKC may be involved in photic [12,16] as well as non-photonic phase shifting [2] effects on the circadian clock in addition to mediating the effects of melatonin [11,20]. These seemingly broad and sometimes conflicting effects of PKC may be the result of different pathways using different PKC isoforms that may be obscured by the use of general PKC activators and inhibitors in some of these studies. To what extent Ca\textsuperscript{2+}-dependent PKC isoforms are expressed in neuronal phenotypes other than AVP neurons or whether they receive input from neuronal populations that contain other neuropeptides is currently unknown.

PKC also modulates neurotransmitter release presynaptically [19] and therefore PKCα and PKCβI may directly influence AVP release from PKCα and/or PKCβI-positive AVP neurons in the SCN. In AVP-negative PKCβI and PKCα cells, release of other neurotransmitters/peptides such as GABA might be modulated by presynaptic PKC. Since on average each PKCβI-positive cell in the small nest-builder SCN contains more PKCβI than in the big nest-builders, neurotransmitter release from PKCβI cells might be differentially regulated among the selected lines. The results in the 10-day-old pups indicate that the expression of PKCβI in the SCN has already reached adult levels at this age, whereas the expression of AVP is apparently still maturing. This discrepancy between PKCβI and AVP expression levels could indicate that these markers might not be functionally linked at this stage of development. Whether the expression of PKCβI in the SCN of 10-day-old pups may be connected to the opening of the eyes at this age remains to be elucidated.

PKCα immunoreactivity in the SCN was not different among the selection lines, and this shows that the difference of AVP in the SCN among the lines is selective in terms of which parts of the putative AVP signal transduction pathway was changed by selection. If the PKCα neuronal population represents the primary AVP receptive neuronal population in the SCN, the higher number of AVP neurons in the SCN of the small nest-builders [8] might not indicate a higher impact on AVP signal transduction. Rather, it could reflect higher levels of AVP content but less AVP release as recently observed in organotypic SCN cultures of rhythmic and non-rhythmic common voles [13]. Indeed, the small nest-builders revealed significantly lower release of AVP per AVP neuron from organotypic SCN slice cultures than the big nest-builders. However, the total release of AVP from these cultures was not significantly different between the selected lines, although on average AVP release was 34% higher in the big nest-builders compared to the small nest-builders (E.A. Van der Zee, D.R. Van der Veen, K. Jansen, M.P. Gerkema, A. Bult, unpublished data).

The differences in circadian rhythmicity among the selected lines cannot be ascribed to PKCβII, since this isoform revealed only low levels of expression and no differences among the lines. PKCβII immunoreactivity in the SCN has been identified in the rabbit and house mouse at moderate to low levels, respectively [7,30], and at very high levels in the white-toothed shrew (Crocidura russula; E.A. Van der Zee, unpublished data) but is absent in the hamster and Arvicanthis niloticus SCN [7]. This heterogeneity among species may suggest different functional roles for PKCβII. Nevertheless, the mice bidirectionally selected for nest-building behavior can be a prominent model system for the investigation of the role of PKCβI and the possible interactions of AVP and PKCα/PKCβI in the regulation of circadian time keeping. We will use pharmacological and physiological approaches to test the proposed functional relationships discussed above.

Bi-directional selection for thermoregulatory nest-building behavior resulted in genetic correlations for expression of circadian locomotor activity patterns and specific aspects of SCN neuroanatomy (Refs. [1,3,8]; this study). Moreover, additional behavioral phenotyping of the two selection lines revealed a striking difference in the way these mice interact with their environment. The big nest-builders actively interact with and change their environ-
ment, e.g., high levels of nest-building, aggressive, and exploratory behaviors), in contrast to the small nest-builders which employ passive coping strategies (Refs. [6, 25]; unpublished data). Hence, the differences in nest-building behavior and organization of circadian rhythmicity could both be unrelated features of the underlying behavioral strategies, which were a consequence of selection for one component of the behavioral strategy, i.e., thermoregulatory nest-building behavior. Since the SCN is the circadian clock and controls circadian locomotor activity [32], we discussed the significance of our findings in this context. The question whether nest-building behavior and circadian function are functionally connected remains to be answered. We are currently performing SCN lesions and SCN transplants to test whether the SCN is directly involved in regulating the amplitude of nest-building behavior.

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