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The role of the neuropeptides PACAP and VIP in the photic regulation of gene expression in the suprachiasmatic nucleus

Joanna M. Dragich,* Dawn H. Loh, Louisa M. Wang, Andrew M. Vosko, Takashi Kudo, Takahiro J. Nakamura, Irene H. Odom, Sei Tateyama, Arkady Hagopian, James A. Waschek and Christopher S. Colwell

Department of Psychiatry and Biobehavioral Sciences, University of California – Los Angeles, 760 Westwood Plaza, Los Angeles, CA 90024 1759, USA

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Abstract

Previously, we have shown that mice deficient in either vasoactive intestinal peptide (VIP) or pituitary adenylate cyclase-activating polypeptide (PACAP) exhibit specific deficits in the behavioral response of their circadian system to light. In this study, we investigated how the photic regulation of the molecular clock within the suprachiasmatic nucleus (SCN) is altered by the loss of these closely-related peptides. During the subjective night, the magnitude of the light-induction of FOS and phosphorylated mitogen-activated protein kinase (p-MAPK) immunoreactive cells within the SCN was significantly reduced in both VIP- and PACAP-deficient mice when compared with wild-type mice. The photic induction of the clock gene *Period1* (*Per1*) in the SCN was reduced in the VIP- but not in the PACAP-deficient mice. Baselines levels of FOS, p-MAPK or *Per1* in the night were not altered by the loss of these peptides. In contrast, during the subjective day, light exposure increased the levels of FOS, p-MAPK and *Per1* in the SCN of VIP-deficient mice, but not in the other genotypes. During this phase, baseline levels of these markers were reduced in the VIP-deficient mice compared with untreated controls. Finally, the loss of either neuropeptide reduced the magnitude of the light-evoked increase in *Per1* levels in the adrenals in the subjective night without any change in baseline levels. In summary, our results indicate that both VIP and PACAP regulate the responsiveness of cells within the SCN to the effects of light. Furthermore, VIP, but not PACAP, is required for the appropriate temporal gating of light-induced gene expression within the SCN.

Introduction

In mammals, the part of the nervous system responsible for most circadian behavior can be localized to a pair of structures in the hypothalamus known as the suprachiasmatic nucleus (SCN). Neurons within the SCN generate robust, synchronized rhythms in the transcription, translation and degradation of key 'clock genes' in an autoregulatory loop that has an endogenous periodicity of approximately 24 h (Reppert & Weaver, 2001; Ko & Takahashi, 2006). Functionally, the molecular clockwork must be synchronized to the external environment. The dominant environmental cue responsible for this synchronization or entrainment is the daily cycle of light and dark. The question of how photic cues from the environment regulate this molecular feedback loop in the SCN is one of the critical issues in the field of circadian rhythms.

The SCN receives photic information directly through a monosynaptic projection from the retina known as the retinohypothalamic tract (RHT). The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are two closely related members of the secretin family (reviewed in Vaudry *et al.*, 2000) that are both expressed in this sensory circuit. PACAP has emerged as a likely transmitter at the RHT/SCN synaptic connection (Hannibal *et al.*, 2000, 2002). Many of the retinal recipient neurons within the ventral SCN express the neuropeptide VIP. Thus, the VIP-expressing cells in the ventral SCN directly receive photic information from the RHT. These retino-recipient cells must then convey this environmental information to the rest of the SCN. Receptors sensitive to these peptides (PAC_1R and $VPAC_2R$) are highly expressed in the SCN (Sheward *et al.*, 1995; Cagampang *et al.*, 1998a,b; Kalamatianos *et al.*, 2004; Kallo *et al.*, 2004). Furthermore, the circadian system of mice deficient in PAC_1R (Hannibal *et al.*, 2001, 2008), $VPAC_2R$ (Harmar *et al.*, 2002), PACAP (Kawaguchi *et al.*, 2003; Beaulé *et al.*, 2009) and VIP each exhibited altered behavioral responses to light. In our own work, we have found that PACAP- and VIP-deficient mice exhibit a loss in the magnitude of light-induced phase advances and delays (Colwell *et al.*, 2003, 2004). In order to better understand the role of these peptides in entrainment, we investigated the impact of the loss of VIP and PACAP on light-induced cellular events within the SCN and its peripheral target, the adrenal gland. We tested the

Correspondence: Dr C. S. Colwell, as above.
E-mail: ccolwell@mednet.ucla.edu

**Present address*: Department of Neurology, Columbia University, 650 West 168th Street, New York City, NY 10032, USA

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hypothesis that the molecular responses of SCN neurons to photic stimulation would be reduced in the VIP- and PACAP-deficient mice.

Materials and methods

Experimental animals

Our studies utilized 2–4-month-old male *Vip* −/− (Colwell *et al.*, 2003) and *Adcyap* (PACAP) −/− (Colwell *et al.*, 2004) mice on a C57BL/6J background (backcrossed for 12 generations). Wild-type (WT) littermates were used when available, but age-matched controls were obtained when necessary. All mice were housed in cages within light-tight chambers with controlled lighting conditions. The experimental protocols used in this study were approved by the UCLA Animal Research Committee, and all recommendations for animal use and welfare, as dictated by the UCLA Division of Laboratory Animals and the guidelines from the National Institutes of Health, were followed.

Behavioral analysis

Male mice of at least 10 weeks of age were housed individually, and their wheel-running activity was recorded as revolutions per 3-min interval. The running wheels and data acquisition system were obtained from Mini Mitter (Bend, OR, USA). The mice were exposed to 12 : 12 h light–dark (LD) for 2–3 weeks (light intensity ≅ 700 lux). The mice were then placed into constant dark (DD) for 4–6 days to assess their free-running activity pattern. The locomotor activity rhythms of mice were analysed by periodogram analysis combined with a χ^2 test with 0.1% significance level (El Temps, Barcelona, Spain) on the raw data. Slopes of an eye-fitted line through the onsets were also used to confirm period estimates made with the periodogram analysis. In order to estimate activity onset on the day of light treatment, a linear regression through 4–6 cycles of onset of activity was calculated. As is standard in this field (for reviews, see Johnson *et al.*, 2003; Roenneberg *et al.*, 2003), circadian time (CT) was determined by activity records, with activity onset denoted as CT12. The endogenous cycle length of each animal is taken into consideration (1 h of CT is equal to Tau/24 * 60 min). About 10% of the VIP-deficient mice did not exhibit a clear onset and could not be used in these experiments. While 4–6 days in DD is not sufficient time to accurately measure free-running period, the periodograms did indicate that the period of the VIP-deficient mice was very similar to our previously published value of 22.5 ± 0.1 h (Colwell *et al.*, 2003), while the PACAP-deficient mice used in the present study were similar to our previously published values of 23.3 ± 0.09 (Colwell *et al.*, 2004). For comparison, WT C57 mice from our colonies exhibit a period of 23.7 ± 0.1 h (n = 28). In the dark portion of LD conditions, as well as under DD conditions, handling of mice was carried out with the aid of an infrared viewer (FJW Industries, OH, USA).

Immunohistochemistry (IHC)

Mice were anesthetized with isoflurane and perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were dissected, post-fixed at 4°C overnight and cryoprotected in 30% sucrose in PBS. IHC was performed on free-floating 30-μm coronal brain sections. Sections were washed for 5 min with PBS (2×) and incubated in 2% H_2O_2 in PBS. Sections were then washed again in PBS (3×), then placed in 10% normal goat serum in PBS for 2 h, then incubated with the primary rabbit antisera in PBS at 4°C overnight. Two antisera were used for these studies: anti-FOS (1 : 30 000; Merck, Darmstadt, Germany) and anti-phosphorylated mitogen-activated protein kinase (pMAPK; 1 : 200; Cell Signaling Technologies, Danvers, MA, USA). Sections were washed in PBS (3×), then incubated with biotinylated goat anti-rabbit antibody 1 : 2000 for 2 h. Sections were washed again for 10 min in PBS (3×) and dipped in AB solution (Vector Labs, Burlingame, CA, USA) for 1 h, washed again in PBS, then placed in filtered 0.05% 3,3′-diaminobenzidine in PBS containing 1 : 10 000 30% H_2O_2. After sufficient color reaction (2–3 min), sections were washed with PBS and mounted on slides immediately. Sections were then dried overnight, washed with water for 10 min, dehydrated with ascending concentrations of ethanol, and cover-slipped.

We defined the SCN using Cresyl violet-stained mouse brains as a reference. For each mouse, images were captured from each of three regions (rostral, central and caudal SCN) using a SPOT camera system (Diagnostic Instruments, Sterling Heights, MI, USA). Three tissue sections from each SCN (rostral, central, caudal aspects of SCN) were chosen and images were taken. The representative sections were obtained from at least three animals. All immunopositive cells within the SCN of these regions were counted manually at 400× with the aid of a grid (200 × 400 μm). All immunopositive cells within the grid were counted equally without regard to the intensity of the staining. Counts were done by two observers blind to treatment protocol and the results averaged.

In situ *hybridization (ISH)*

A plasmid (pCRII; Invitrogen, Carlsbad, CA, USA) containing the cDNA for *Per1* (340–761 nt, accession number AF022992) was generously provided by Dr D. Weaver (Univ. Mass.), and insert identity was confirmed by sequencing using the M13R primer. To generate antisense and sense templates for ISH, plasmids were linearized overnight, phenol : chloroform extracted, ethanol precipitated and resuspended in DEPC-treated water. Riboprobes were synthesized from 1 μg of template cDNA in a reaction mixture containing 100 μCi of UTP ^{35}S (1250 Ci/mmol; Perkin Elmer, Wellesley, MA, USA), 5× transcription buffer (Promega, Madison, WI, USA), 0.1 M dithiothreitol (DTT; Promega), 10 mM of each rATP, rCTP, rGTP, 40 U RNase Inhibitor, and the appropriate RNA transcriptase (SP6, or T7) for 3 h at 37°C. The *in vitro* transcription reaction was DNase I treated, then unincorporated nucleotides were removed using the RNase-free microfuge spin columns (Bio-Spin 30; Biorad, Hercules, CA, USA) and probe yields were calculated by scintillation counting. ISH on tissue sections was done using previously described procedures (Chaudhury *et al.*, 2008; Wang *et al.*, 2009). Briefly, mice were anesthetized with isoflurane and brains were removed, immediately frozen in embedding media (OTC; Sakura Finetek, Torrance, CA, USA) and stored at −80°C. Sections were taken at 20 μm thickness in the coronal plane and mounted onto superfrost plus slides (Fisher Scientific, Pittsburgh, PA, USA). For each genotype, time-matched controls were sectioned onto the same slide as mice exposed to a light pulse. On Day 1, slides were warmed to room temperature, briefly washed in PBS and fixed in 4% paraformaldehyde, air-dried and blocked by acetylation with acetic anhydride, followed by a series of dehydration steps. After air drying, slides were placed in prehybridization buffer [50% formamide, 3 M NaCl, 20 mM EDTA, 400 mM Tris, pH 7.8, 0.4% sodium dodecyl sulfate (SDS), 2× Denhardt's, 500 mg/mL tRNA and 50 mg/mL polyA RNA] for 1 h at 55°C. Sections were then hybridized overnight at 55°C in humidified chambers in hybridization buffer (50% formamide, 10% dextran sulfate, 3 M NaCl, 20 mM EDTA, 400 mM

Tris, pH 7.8, 0.4% SDS, 2× Denhardt's, 500 mg/mL tRNA, 50 mg/mL polyA RNA and 40 mM DTT), where each slide was incubated with 1–4 million cpm/70 mL of a riboprobe. Following hybridization, the slides were washed for 15 min in 4× standard sodium citrate (SSC), at their respective hybridization temperatures, in 2× SSC for 1 h at room temperature, then RNase A (20 μg/mL) treated at 37°C for 30 min to remove unbound probe. To further reduce non-specific hybridization, the slides were washed twice in 2× SSC at 37°C, and for 1 h in 0.1× SSC at 62–67°C. Slides were serially dehydrated in ethanol containing 0.3 M ammonium acetate and exposed to Kodak Biomax MR film (Kodak, Rochester, NY, USA) along with a ^{14}C slide standard (American Radiolabeled Chemicals, St Louis, MO, USA). The slides were counterstained with Cresyl violet to serve as a reference. Densitometric analysis of hybridization intensity was done as described using NIH image software (Shearman *et al.*, 1997; Chaudhury *et al.*, 2008; Wang *et al.*, 2009).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from adrenal glands using the Trizol (Invitrogen) procedure and treated with DNase (Turbo DNA-free, Ambion, Austin, TX, USA) for 30 min to remove possible DNA contaminants. cDNA was obtained by reverse-transcription of 1 μg total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Austin, TX, USA) and no-reverse transcriptase (RT) controls were run routinely. Primers for *Period1* (*Per1*) and *Actb* were designed using Oligo6 (Molecular Biology Insights, Cascade, CO, USA) and Mfold programs to cross intron–exon boundaries and hence further prevent contaminants in the subsequent RT-PCR. Oligonucleotide primer sequences used were *Per1*-sense: 5′-TCC TCC TCC TAC ACT GCC TCT-3′ and *Per1*-antisense: 5′-TTG CTG ACG ACG GAT CTT T-3′; and *Actb*-sense: 5′-CCA ACC GTG AAA AGA TGA CC-3′ and *Actb*-antisense: 5′-CCA GAG GCA TAC AGG GAC AG-3′. PCR products from each primer pair were cloned and sequenced to confirm specificity as well as run on an agarose gel to ensure no primer dimers were formed. Quantitative PCR using SYBR Green (SYBR Green PCR Master Mix, Applied Biosystems) was performed using the equivalent of 50 ng of starting total RNA (1 : 20 of the initial cDNA reaction from 1 μg of total RNA) in a 25-μL reaction comprising of the 2× SYBR Green reaction mix and 1.2 μM each of the forward and reverse primers. The efficiency of each primer pair was confirmed prior to and during experimental use by serial dilutions of a cDNA sample, and by plotting C_t (cycle number at which threshold within the linear range was reached) values against log(total RNA). Melting curves were determined at the end of each reaction to check product specificity. The relative levels of *Per1* transcripts were determined using the $2^{-\Delta\Delta Ct}$ method, using *Actb* as the normalizing reference gene. We confirmed that the levels of *Actb* did not vary with the daily cycle.

Statistical measurements

The data sets were analysed by a two-way analysis of variance (ANOVA), with genotype and light exposure as the factors. If significant group differences were detected ($P < 0.05$) by the ANOVA, then the Holm–Sidak method for pair-wise multiple comparison was used. In the cases in which the baseline levels were found to significantly vary between genotype, we also examined the fold induction due to light exposure using one-way ANOVA. If the data did not show equal variance or normal distribution, then a Kruskal–Wallis one-way ANOVA on ranks was used. For all tests, values were considered significantly different if $P < 0.05$. All tests were performed using SIGMASTAT (version 3.5, Systat Software, San Jose, CA, USA). Values are shown as mean ± SEM.

Results

Loss of VIP and PACAP alters the photic regulation of FOS expression in the SCN

The light-induction of FOS in the SCN is one of the most robust and well-characterized markers of the photic regulation gene expression in the SCN (e.g. Kornhauser *et al.*, 1990). Therefore, in order to determine if the light-induced translational response within the SCN is impaired in VIP- or PACAP-deficient mice, we used IHC to measure the number of FOS-immunopositive cells (FOS+) within the SCN. Mice were held in DD and wheel-running activity measured to determine circadian phase (Fig. 1). While all of the VIP-deficient mice exhibit disruptions in their ability to express a coherent circadian rhythm in constant conditions (Colwell *et al.*, 2003; Aton *et al.*, 2005; Vosko *et al.*, 2007; Ciarleglio *et al.*, 2009), most remain rhythmic for a few weeks in DD conditions. Experimental mice were then exposed to light (white light, 1.5×10^{-1} μW/cm^2; 10 min duration) at one of three phases of the daily cycle and tissue collected 60 min after the beginning of the light treatment. Tissue from time-matched control mice was also collected for comparison. Control experiments in which the primary antibody was not added, or the primary antibody was pre-absorbed with a peptide control, did not exhibit any positive staining (data not shown).

At CT23, the two-way ANOVA detected significant variation between genotypes ($F_{2,21} = 23.46$, $P = 0.001$), light treatment ($F_{1,21} = 215.91$, $P = 0.001$), as well as a significant interaction between genotype and light ($F_{2,21} = 20.33$, $P = 0.001$). Further *post hoc* analysis of these data indicated that all three genotypes (WT: $t_7 = 13.45$, $P = 0.0001$; VIP −/−: $t_7 = 3.77$, $P = 0.002$; PACAP −/−: $t_6 = 8.61$, $P = 0.0001$) displayed significant increases in the number of FOS+ cells in the SCN after exposure to light at CT23 when compared with untreated controls (Fig. 2A and B). The magnitude of this light-induction was significantly reduced in both VIP- ($t_7 = 9.73$, $n = 4$, $P = 0.0001$) and PACAP- ($t_7 = 4.45$, $n = 4$, $P = 0.0001$) deficient mice when compared with WT mice ($n = 4$). There were no significant differences between the baseline levels of FOS+ cells at CT23 in VIP- ($t_6 = 0.322$, $n = 3$, $P = 0.751$) or PACAP-deficient ($t_6 = 0.275$, $n = 3$, $P = 0.787$) mice compared with WT controls ($n = 4$).

At CT16, the two-way ANOVA detected significant variation between genotypes ($F_{2,34} = 21.14$, $P = 0.001$) and light treatment ($F_{1,34} = 274.63$, $P = 0.001$), as well as a significant interaction between genotype and light ($F_{2,34} = 10.26$, $P = 0.001$). *Post hoc* analysis of these data indicated that all three genotypes (WT: $t_{13} = 14.20$, $P = 0.0001$; VIP −/−: $t_8 = 6.46$, $P = 0.0001$; PACAP −/−: $t_{11} = 8.81$, $P = 0.0001$) displayed significant increases in the number of FOS+ cells in the SCN after exposure to light at CT16 when compared with untreated controls (Fig. 2C and D). The magnitude of this induction was significantly reduced in both VIP- ($t_9 = 7.25$, $n = 5$, $P = 0.0001$) and PACAP- ($t_{10} = 4.9$, $n = 5$, $P = 0.0001$) deficient mice when compared with WT mice ($n = 6$). There were no significant differences between the baseline levels of FOS+ cells at CT16 in VIP- ($t_{12} = 1.661$, $n = 4$, $P = 0.107$) or PACAP-deficient ($t_{14} = 0.264$, $n = 6$, $P = 0.794$) mice compared with WT controls ($n = 9$).

At CT6, the two-way ANOVA detected no significant variation between genotypes ($F_{2,20} = 0.174$, $P = 0.842$), while significant differences between light treatment ($F_{1,20} = 26.86$, $P = 0.001$) as well

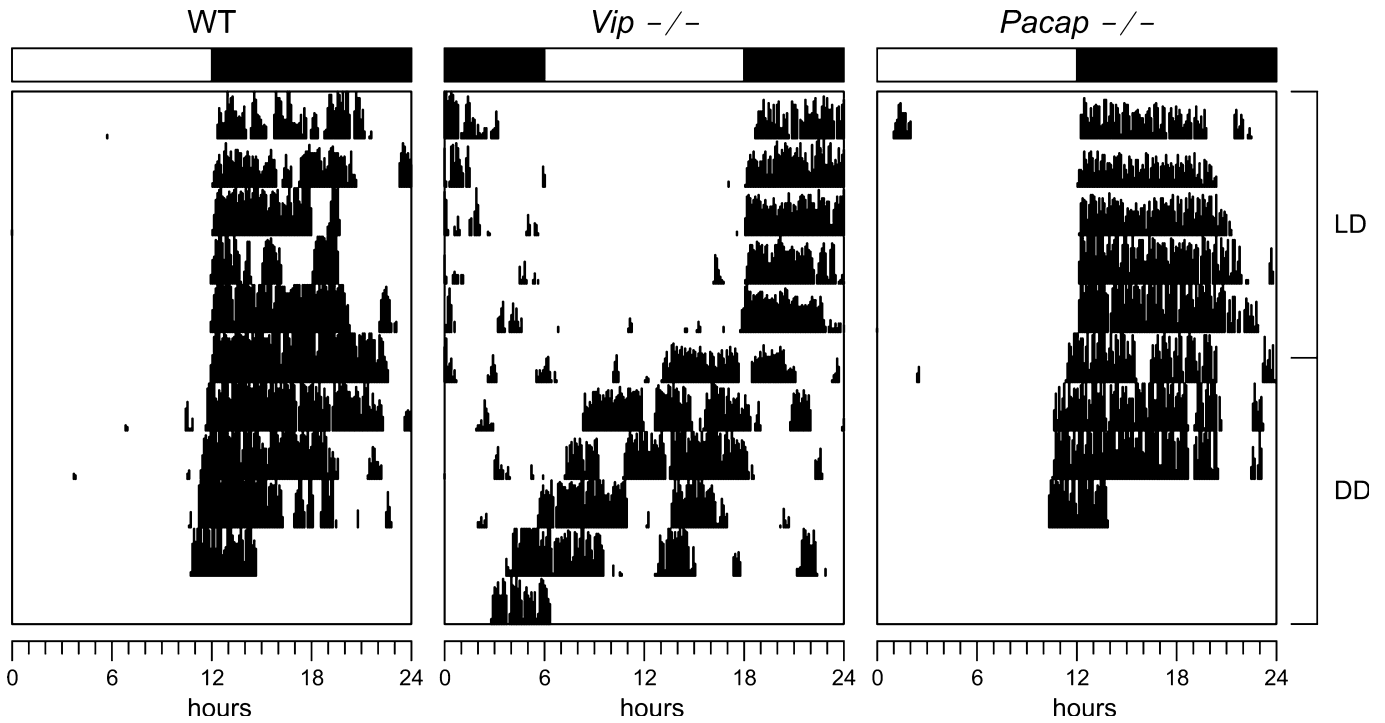


FIG. 1. Examples of wheel-running activity of mice from each genotype. In order to determine activity onset, mice were placed in cages with access to running wheels and wheel-running activity was recorded. Typically, the mice were held in light–dark (LD) conditions for 14 days prior to being released in constant darkness (DD) for 4–6 days prior to being exposed to light at CT16. CT was determined by activity records, with activity onset denoted as CT12. In order to estimate activity onset on the day of light treatment, a linear regression through four-six cycles of onset of activity was calculated. The endogenous cycle length of each animal was taken into consideration (1 h of CT is equal to Tau/24 * 60 min). Calculation of CT requires clear activity onsets, and approximately 10% of the vasoactive intestinal peptide (VIP)-deficient mice could not be used for this analysis, compared with 5% of the pituitary adenylate cyclase-activating polypeptide (PACAP)-deficient and wild-type (WT) mice. Examples of wheel-running activity from WT (C57 BL/6J, left panel), VIP-deficient (*Vip* −/− BL/6J, middle panel) and PACAP-deficient (*Adcyap* −/− BL/6J, right panel) mice are shown. Bars at the top of each panel indicate the LD cycle prior to DD.

as a significant interaction between genotype and light ($F_{2,20} = 16.25$, $P = 0.001$) were found. *Post hoc* analysis of this indicated that VIP-deficient ($t_6 = 7.65$, $P = 0.0001$) but not WT ($t_7 = 0.98$, $P = 0.34$) or PACAP-deficient ($t_5 = 0.39$, $P = 0.69$) mice displayed significant increases in the number of FOS+ cells in the SCN after exposure to light at CT6 when compared with untreated controls (Fig. 2E and F). The number of FOS+ cells was significantly higher in the light-treated VIP-deficient ($t_6 = 3.34$, $n = 4$, $P = 0.004$) but not PACAP-($t_6 = 0.64$, $n = 3$, $P = 0.53$) deficient mice when compared with WT mice ($n = 4$). Baseline levels of FOS+ cells at CT6 were significantly reduced in the VIP-deficient mice ($t_6 = 3.64$, $P = 0.002$) when compared with WT mice (Fig. 2F). Because baseline levels changed, we also compared the light-evoked fold-increase in the number of FOS+ cells in the SCN. With this measure, there were significant differences (Kruskal–Wallis ANOVA on ranks: $H_{2,9} = 5.98$, $P = 0.034$) between the genotypes (WT: 1.36 ± 0.3, $n = 4$; VIP: 61.11 ± 14.1, $n = 3$; PACAP: 1.11 ± 0.1, $n = 3$), with the VIP-deficient mice exhibiting a robust fold-induction.

Loss of VIP and PACAP alters the photic activation of p-MAPK in the SCN

Previous work suggests that photic stimulation also results in the activation of the MAPK signaling pathway in SCN neurons (e.g. Obrietan *et al.*, 1998; Butcher *et al.*, 2002, 2005). Therefore, we used an antibody directed against the phosphorylated form of MAPK (p-MAPK) to determine if the light-activation of this signaling pathway is also impaired in the SCN of VIP- or PACAP-deficient mice. Mice were held in DD and wheel-running activity was measured to determine circadian phase. Experimental mice were then exposed to light (see above) at CT16 and tissue was collected 30 min after the beginning of the light treatment. Tissue from time-matched control mice was also collected for comparison. Control experiments in which the primary antibody was not added or the primary antibody was pre-absorbed with a peptide control did not exhibit any positive staining (data not shown).

At CT16, the two-way ANOVA detected significant variation between genotypes ($F_{2,25} = 5.31$, $P = 0.014$) and light treatment ($F_{1,25} = 33.28$, $P = 0.001$), as well as a significant interaction between genotype and light ($F_{2,25} = 9.82$, $P = 0.001$). *Post hoc* analysis of these data indicated that WT ($t_9 = 7.48$, $P = 0.0001$) but not VIP-($t_9 = 2.02$, $P = 0.057$) or PACAP-deficient ($t_5 = 1.19$, $P = 0.245$) mice displayed significant increases in the number of p-MAPK+ cells in the SCN after exposure to light at CT16 when compared with untreated controls (Fig. 3A and B). The number of p-MAPK+ cells was significantly reduced in the light-treated VIP- ($t_9 = 4.92$, $n = 5$, $P = 0.0001$) and PACAP- ($t_7 = 4.17$, $n = 3$, $P = 0.0001$) deficient mice when compared with WT mice ($n = 5$). There were no significant differences between the baseline levels of p-MAPK+ cells at CT16 in VIP- ($t_9 = 0.542$, $n = 5$, $P = 0.594$) or PACAP-deficient ($t_7 = 0.542$, $n = 3$, $P = 0.343$) mice compared with WT controls ($n = 5$).

At CT6, the two-way ANOVA detected no significant variation between genotypes ($F_{1,13} = 3.66$, $P = 0.085$) and light treatment ($F_{1,13} = 2.93$, $P = 0.118$), but a significant interaction between genotype and light ($F_{2,13} = 19.03$, $P = 0.001$). PACAP-deficient mice were not examined at this phase. *Post hoc* analysis of these data (Holm–Sidak multiple comparison procedures) indicated that VIP-deficient ($t_7 = 4.64$, $P = 0.001$) but not WT ($t_5 = 1.75$, $P = 0.110$) mice

displayed significant increases in the number of p-MAPK+ cells in the SCN after exposure to light at CT6 when compared with untreated controls (Fig. 3C). Baseline levels of p-MAPK+ cells at CT6 were significantly reduced in the VIP-deficient mice ($t_6 = 4.44$, $P = 0.001$) when compared with WT mice (Fig. 3C). Because baseline levels changed, we also compared the light-evoked fold-increase in the number of p-MAPK+ cells in the SCN. With this measure, there were significant differences (ANOVA: $F_{1,6} = 30.62$, $P = 0.003$) between the genotypes (WT: 0.81 ± 0.12, $n = 3$; VIP: 2.24 ± 0.22, $n = 4$), with the VIP-deficient mice exhibiting a robust fold-induction.

Loss of VIP and PACAP alters the photic induction of Period message in the SCN

Photic activation of the clock gene, *Per1*, is likely to be critical for the entrainment of the molecular clock within SCN neurons (e.g. Shigeyoshi *et al.*, 1997; Akiyama *et al.*, 1999; Wakamatsu *et al.*, 2001), and we sought to examine whether the photic induction of the message coding for *Per1* is disrupted in the mutants. We used ISH to measure *Per1* message in the SCN. Mice were held in DD and wheel-running activity was measured to determine circadian phase. Experimental mice were then exposed to light (see above) at one of three phases of the daily cycle and tissue was collected 60 min after the beginning of the light treatment. Tissue from time-matched control mice was also collected for comparison. The *Per1* sense probe did not exhibit labeling in the brain under identical hybridization conditions (data not shown).

At CT23, the two-way ANOVA detected no significant variation between genotypes ($F_{2,21} = 0.29$, $P = 0.75$), while significant difference between light treatment was found ($F_{1,21} = 65.02$, $P = 0.001$). There was not a significant interaction between genotype and light ($F_{2,21} = 3.26$, $P = 0.065$). Further *post hoc* analysis of these data indicated that all three genotypes (WT: $t_7 = 5.86$, $P = 0.0001$; VIP −/−: $t_7 = 2.76$, $P = 0.014$; PACAP −/−: $t_5 = 5.28$, $P = 0.0001$) displayed significant increases in the number of *Per1* message in the SCN after exposure to light at CT23 when compared with untreated controls (Fig. 4A and B). The magnitude of this light-induction was not significantly reduced in either VIP- ($t_7 = 1.27$, $n = 4$, $P = 0.223$) or PACAP- ($t_6 = 0.38$, $n = 3$, $P = 0.706$) deficient mice when compared with WT mice ($n = 4$). There were no significant differences between the baseline levels of *Per1* at CT23 in VIP- ($t_7 = 1.267$, $n = 4$, $P = 0.223$) or PACAP-deficient ($t_6 = 1.009$, $n = 3$, $P = 0.328$) mice compared with WT controls ($n = 4$).

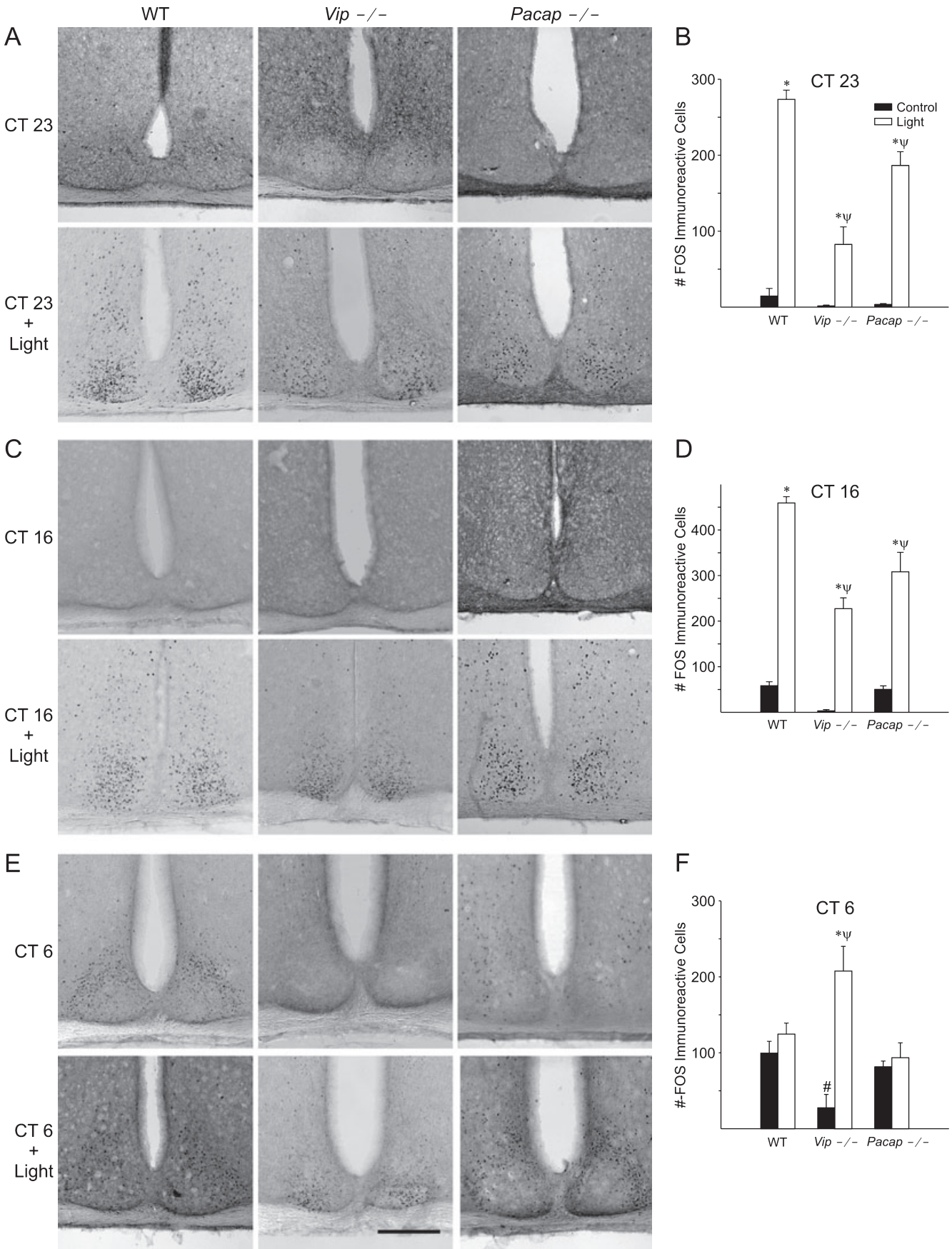
At CT16, the two-way ANOVA detected significant variation between genotypes ($F_{2,21} = 6.0$, $P = 0.011$) and light treatment ($F_{1,21} = 86.86$, $P = 0.001$), as well as a significant interaction between genotype and light ($F_{2,21} = 6.17$, $P = 0.01$). Further *post hoc* analysis of these data indicated that all three genotypes (WT: $t_9 = 6.77$, $P = 0.0001$; VIP −/−: $t_5 = 2.42$, $P = 0.028$; PACAP −/−: $t_5 = 7.37$, $P = 0.0001$) displayed significant increases in the number of *Per1* message in the SCN after exposure to light at CT16 when compared with untreated controls (Fig. 4C and D). The magnitude of this light-induction was significantly reduced in VIP- ($t_7 = 2.76$, $n = 3$, $P = 0.014$) and enhanced in PACAP- ($t_7 = 2.73$, $n = 3$, $P = 0.015$) deficient mice when compared with WT mice ($n = 5$). There were no significant differences between the baseline levels of *Per1* at CT16 in VIP- ($t_7 = 0.393$, $n = 3$, $P = 0.699$) or PACAP-deficient ($t_7 = 0.355$, $n = 3$, $P = 0.728$) mice compared with WT controls ($n = 5$).

At CT6, the two-way ANOVA detected significant variation between genotypes ($F_{2,21} = 13.816$, $P = 0.001$) and a significant interaction between genotype and light ($F_{2,21} = 4.095$, $P = 0.037$), but no significant variation between light treatments ($F_{1,21} = 2.295$, $P = 0.149$). *Post hoc* analysis of these data indicated that VIP-deficient ($t_7 = 3.172$, $P = 0.006$) but not WT ($t_7 = 0.732$, $P = 0.475$) or PACAP-deficient ($t_5 = 0.986$, $P = 0.339$) mice displayed significant increases in the levels of *Per1* in the SCN after exposure to light at CT6 when compared with untreated controls (Fig. 4E). However, the magnitude of *Per1* expression in the light-treated groups did not vary in the VIP- ($t_7 = 1.22$, $n = 4$, $P = 0.240$) or PACAP- ($t_6 = 0.490$, $n = 3$, $P = 0.631$) deficient mice when compared with WT mice ($n = 4$). Baseline levels of *Per1* in the SCN at CT6 were significantly reduced in the VIP-deficient mice ($t_7 = 3.661$, $P = 0.002$) and significantly increased in PACAP-deficient ($t_6 = 2.22$, $P = 0.041$) mice (when compared with WT mice (Fig. 4F). Because baseline levels changed, we also compared the light-evoked fold-increase in the levels of *Per1* in the SCN. With this measure, there were significant differences (Kruskal–Wallis ANOVA on ranks: $H_{2,10} = 6.42$, $P = 0.022$) between the genotypes (WT: 1.0 ± 0.7, $n = 4$; VIP: 1.8 ± 0.3, $n = 4$; PACAP: 0.85 ± 0.03, $n = 3$).

Loss of VIP and PACAP alters the photic regulation of Period message in the adrenals

Light also regulates the expression of clock genes in peripheral organs such as the adrenals (Ishida *et al.*, 2005). Therefore, to determine if photic regulation is altered in peripheral organs of VIP- or PACAP-deficient mice, we used real-time PCR to measure the levels of *Per1* transcript in the adrenals. Mice were held in DD and wheel-running activity was measured to determine circadian phase. Experimental mice were then exposed to light (white light, 1.5×10^{-1} μW/cm^2;

FIG. 2. Loss of vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) alters the photic regulation of FOS expression in the SCN. Mice were held in DD, and wheel-running activity was measured to determine circadian phase. Mice were exposed to light (white light, 1.5×10^{-1} μW/cm^2; 10 min duration) at one of three phases of the daily cycle, and tissue was collected 60 min after the beginning of the light treatment. Tissue from time-matched controls was collected for comparison. IHC was used to measure FOS immunoreactivity in the SCN ($n = 3$–9 per group). Possible significant differences between the groups were assessed with a two-way ANOVA followed by Holm–Sidak multiple comparisons procedure. Our criterion level for significance was set at $P < 0.05$, with * indicating significant differences between untreated and light-treated groups, ψ indicating significant differences between light-treated groups; # indicating significance differences between untreated baseline groups. These symbols and statistical test apply to all of the histograms. Error bars in this and all later figures represent SEM. (A) Photomicrographs of SCN tissue of wild-type (WT), VIP-deficient, PACAP-deficient mice exposed to light at circadian time (CT)23 along with time-matched controls. (B) All three genotypes displayed significant increases in the number of FOS+ cells in the SCN after exposure to light at CT23 when compared with untreated controls. The magnitude of this light-induction was significantly reduced in both VIP- and PACAP-deficient mice when compared with WT mice. There were no significant differences between the baseline levels of FOS+ cells at CT23. (C) Photomicrographs of SCN tissue from WT, VIP −/− and PACAP −/− mice exposed to light at CT16 along with time-matched controls. (D) All three genotypes displayed significant increases in the number of FOS+ cells in the SCN after exposure to light at CT16 when compared with untreated controls. The magnitude of this light-induction was significantly reduced in both VIP- and PACAP-deficient mice when compared with WT mice. There were no significant differences between the baseline levels of FOS+ cells at CT16. (E) Photomicrographs of SCN tissue from WT, VIP −/− and PACAP −/− mice exposed to light at CT6 along with time-matched controls. (F) VIP-deficient, but not WT or PACAP-deficient, mice displayed significant increases in the number of FOS+ cells in the SCN after exposure to light at CT6 when compared with untreated controls. The number of FOS+ cells was significantly higher in the light-treated VIP-deficient but not PACAP-deficient mice when compared with WT mice. The baseline number of FOS+ cells at CT6 was reduced in VIP-deficient mice compared with the other genotypes. Scale bar: 200 μm (and applies to all of the photomicrographs).



10 min duration) during the subjective night (CT16) or subjective day (CT6), and tissue was collected 60 min after the beginning of the light treatment. Tissue from time-matched control mice was also collected for comparison.

At CT16, the two-way ANOVA detected significant variation between genotypes ($F_{2,23} = 4.34$, $P = 0.029$) and significant interaction between genotype and light ($F_{2,23} = 4.69$, $P = 0.023$), while no significant differences between light treatments were found ($F_{1,23} = 1.605$, $P = 0.221$). *Post hoc* analysis of these data indicated that WT ($t_8 = 2.988$, $P = 0.008$) but not VIP- ($t_6 = 1.407$, $P = 0.176$) or PACAP-deficient ($t_7 = 0.784$, $P = 0.443$) mice displayed significant increases in the levels of *Per1* in the adrenals after exposure to light at CT16 when compared with untreated controls (Fig. 5A). The magnitude of *Per1* expression in the light-treated groups was significantly reduced in the VIP- ($t_9 = 4.808$, $n = 4$, $P = 0.0001$) but not the PACAP- ($t_{10} = 2.035$, $n = 5$, $P = 0.057$) deficient mice when compared with WT mice ($n = 6$). There were no significant differences between the baseline levels of *Per1* at CT16 in VIP- ($t_5 = 0.103$, $n = 3$, $P = 0.919$) or PACAP-deficient ($t_5 = 0.377$, $n = 3$, $P = 0.710$) mice compared with WT controls ($n = 3$). At CT6, the two-way ANOVA did not detect significant variation between genotypes ($F_{2,29} = 1.932$, $P = 0.167$), between light treatments ($F_{1,29} = 0.159$, $P = 0.693$), or any significant interaction between genotype and light ($F_{2,29} = 0.235$, $P = 0.792$). Therefore, these data were not further analysed (Fig. 5B).

Discussion

In this study, we investigated how the photic regulation of the molecular clock within the SCN, and its peripheral targets, is altered by the loss of PACAP or VIP. One striking finding is that light exposure during the subjective day increased FOS+, p-MAPK+ and *Per1* in the SCN of VIP-deficient mice, but not in the other genotypes. Baseline levels of these markers were also reduced in the VIP-

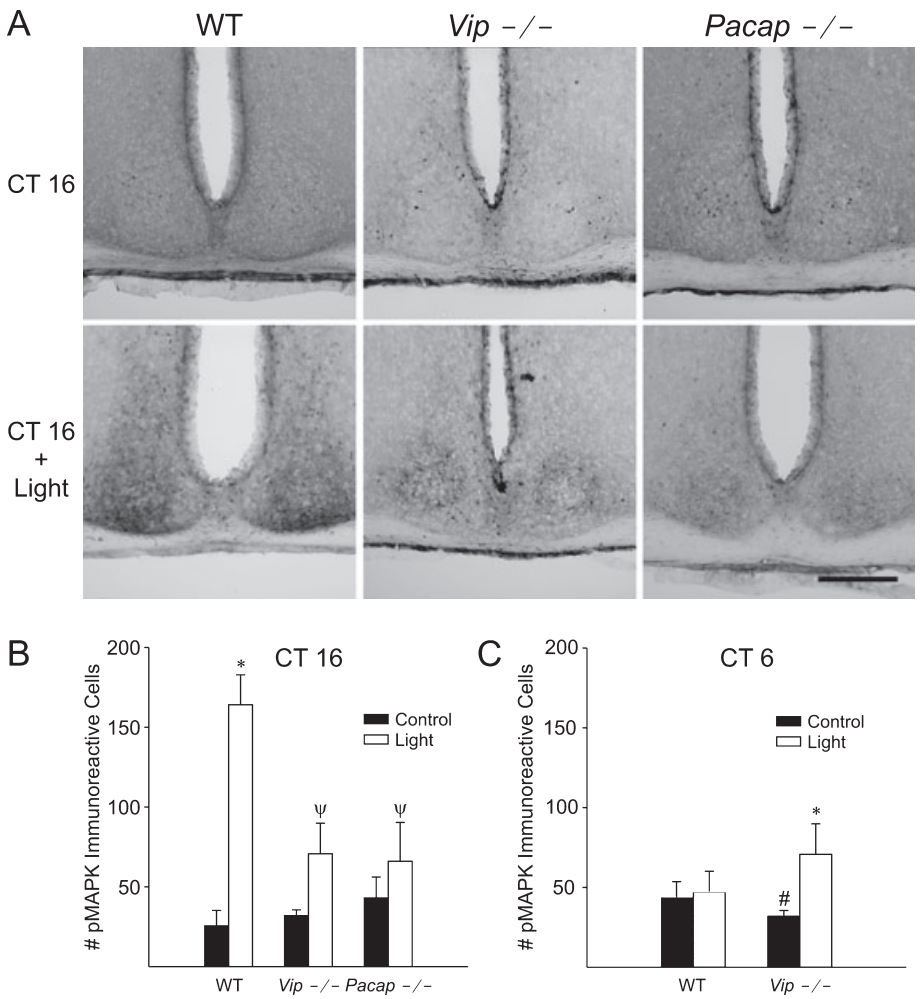


FIG. 3. Loss of vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) alters the photic regulation of phosphorylated mitogen-activated protein kinase (p-MAPK) expression in the SCN. Mice were held in DD and wheel-running activity was measured to determine circadian phase. Mice were exposed to light (see above) at circadian time (CT)16 or CT6, and tissue was collected 30 min after the beginning of the light treatment. Tissue from time-matched controls was collected for comparison. IHC was used to measure p-MAPK immunoreactivity in the SCN ($n = 3$–5 per group). (A) Photomicrographs of SCN tissue from wild-type (WT), VIP −/− and PACAP −/− mice exposed to light at CT16 along with time-matched controls. (B) WT, but not VIP- or PACAP-deficient mice, displayed significant increases in the number of p-MAPK+ cells in the SCN after exposure to light at CT16 when compared with untreated controls. The number of p-MAPK+ cells was significantly reduced in the light-treated VIP- and PACAP-deficient mice when compared with WT mice. There were no significant differences between the baseline levels of p-MAPK+ cells at CT16. (C) VIP-deficient, but not WT, mice displayed significant increases in the number of p-MAPK+ cells in the SCN after exposure to light at CT6 when compared with untreated controls. Baseline levels of p-MAPK+ cells at CT6 were significantly reduced in the VIP-deficient mice when compared with WT mice. The light-evoked fold-increase in the number of p-MAPK+ cells in the SCN was higher in VIP-deficient (2.24 ± 0.22, $n = 4$) than WT (0.81 ± 0.12, $n = 3$) mice. See Fig. 2 legend for symbol definitions.

deficient mice. Another major finding is that both PACAP- and VIP-deficient mice were less responsive to light during the subjective night as measured by the number of FOS+ and p-MAPK+ cells within the SCN. The loss of VIP reduced the magnitude of light-activated *Per1* message in the early night. During the subjective night, baseline levels of FOS, p-MAPK or *Per1* were not altered. Finally, we found that, in the adrenals, the loss of either neuropeptide reduced the magnitude of the light-evoked increase in *Per1* levels in the subjective night compared with WT controls. This provides a clear indication that these peptides can have a different regulatory role in the photic regulation of clock gene expression in the periphery than in the central clock.

Abnormal light-induced gene expression within the SCN during the subjective day

Light normally induces phase shifts of the circadian system during the night but not during the day. Importantly, this phase dependence in behavior is normally mirrored by the light-induced changes in gene expression within the SCN. A variety of studies have found a close association between light-induced changes in behavior and induction of immediate-early genes and clock genes in the SCN (e.g. Kornhauser *et al.*, 1990; Rieux *et al.*, 2002). This 'gating' is a fundamental feature of the photic response of the circadian system (Johnson *et al.*, 2003). Perhaps the most striking finding in the present study was the loss of the normal gating of the light-evoked gene expression in the SCN. During the subjective day, light produced a significant increase in FOS+, p-MAPK+ and *Per1* message in VIP-deficient mice. The baseline levels of these markers were reduced in the VIP mutants, suggesting the need for further analysis of the role of VIP in driving the daytime levels of clock gene expression. Previous work with the *Vipr2* −/− mice reported that light at CT6 caused a significant light-induction of FOS and p-ERK in the SCN during the day (Hughes *et al.*, 2004). These findings suggest a fundamental role for VIP, but not PACAP, in the gating of light input to the SCN.

There are a number of possible explanations for this finding that will require further analysis of the specific cell types in which FOS or *Per1* are induced during the subjective day. For example, it is possible that the gating is lost at the population level but not at the cellular level. As the loss of VIP reduces the synchrony of the SCN cell population (Aton *et al.*, 2005; Brown *et al.*, 2007; Ciarleglio *et al.*, 2009), it is possible that individual cellular oscillators are in a 'night' state even during the subjective day. In this case, gating at the cellular level may be unaffected by the loss of VIP and the light response during the subjective day may be due to the desynchronization of the cell population. Alternatively, VIP through its regulation of cAMP/protein kinase A signaling could be acting at the level of the SCN neuron's membrane or intracellular signaling cascades to prevent gene expression during inappropriate phases of the daily cycle. For example, we (Colwell, 2001) and others (Pennartz *et al.*, 2001) have suggested that part of the gating mechanism involves a nightly upregulation in the magnitude of *N*-methyl-D-aspartate (NMDA)-evoked calcium responses. VIP can be a potent regulator of NMDA currents (Yang *et al.*, 2009) and perhaps, in the absence of VIP, the daily rhythm in the magnitude of NMDA-evoked currents and calcium response is lost. Of course, other elements in this intracellular signaling cascade could be impacted as well. Anatomical analysis and modeling studies of the SCN has led Silver and colleagues to suggest that the SCN contains a population of non-rhythmic 'gate' cells (Antle & Silver, 2005; Antle *et al.*, 2007). The loss of VIP may prevent these gating cells from communicating to the remaining light-responsive cells within the nucleus. Regardless of the mechanistic explanation, the finding that light still does not induce a behavioral response at CT6 (present study; also see Hughes & Piggins, 2008) is also significant. These mutant mice clearly demonstrate a disassociation between light-induced gene expression in the SCN and circadian behavior.

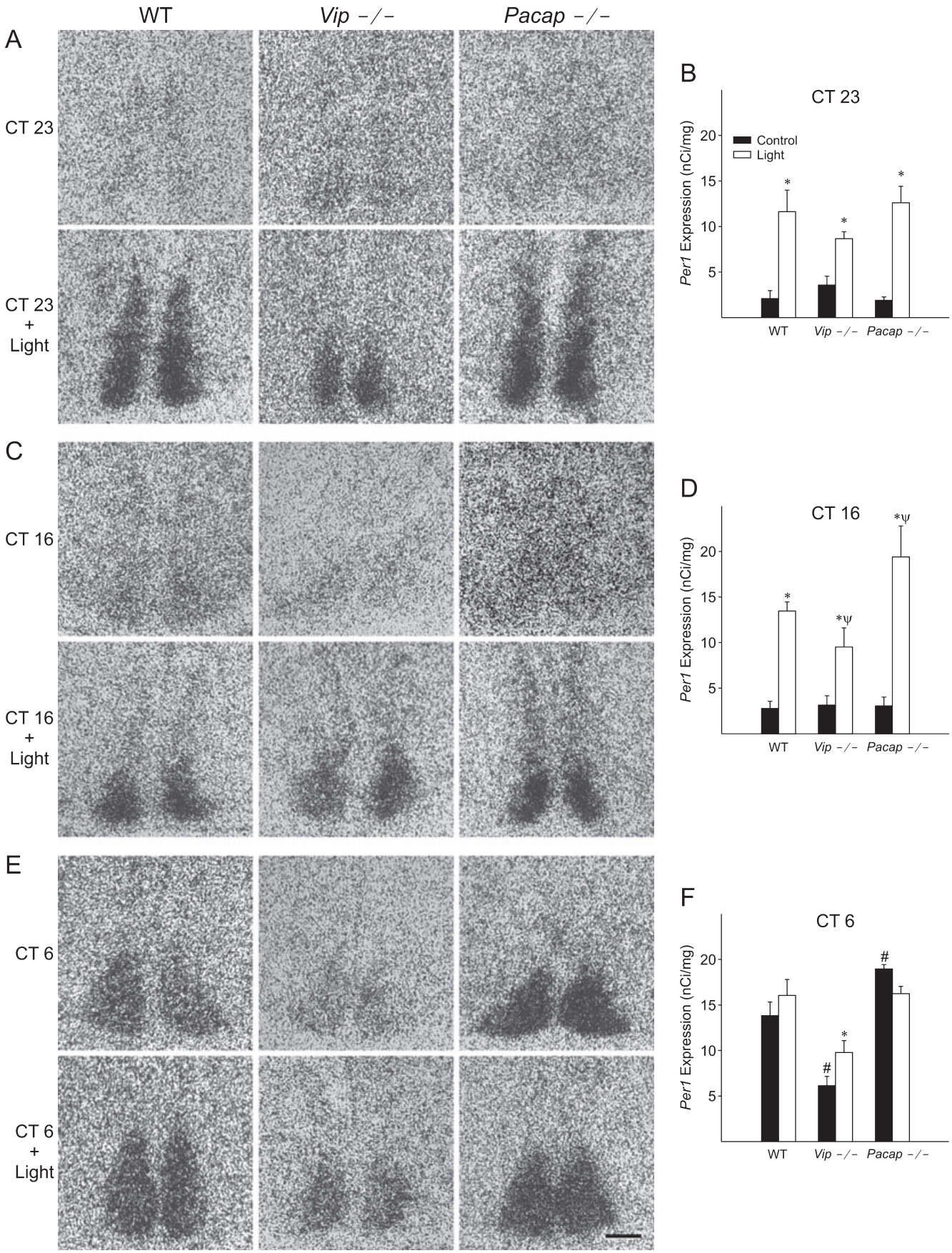
Reduced light-induced gene expression within the SCN during the subjective night

The light-induction of FOS in the SCN is one of the most robust and well-characterized markers of the photically regulated gene expression in the SCN (e.g. Kornhauser *et al.*, 1990), and the MAPK pathway is thought to be a critical component of the signal-transduction cascade by which light regulates immediate-early gene expression (Obrietan *et al.*, 1998; Butcher *et al.*, 2002, 2005; Dziema *et al.*, 2003). In the present study, we found that PACAP-deficient mice were less responsive to light as measured by the photic induction of FOS expression and MAPK phosphorylation within the SCN. The magnitude of light-induction of FOS was modestly reduced in both the early and late night. This work is consistent with previous results with PAC_1R −/− mice (Hannibal *et al.*, 2001) that found a significant reduction in light-induced *c-Fos* message in the early and late night. However, very similar experiments carried out in PACAP-deficient mice on a commonly used outbred line of mice (Institute of Cancer Research, ICR) exhibited a reduction in the magnitude of light-induced FOS in the early but not the late night (Kawaguchi *et al.*, 2003). Furthermore, a recent study on another line of PACAP-deficient mice (C57 background) found that the loss of PACAP did not alter light-induction of FOS in the late night, but did not examine the effect in the early night (Beaulé *et al.*, 2009).

Some of this apparent variability may be explained by differences in the genetic background or extent of the genetic lesion. In particular, our PACAP-deficient mice (Colwell *et al.*, 2004) are also missing the gene coding for a PACAP-related peptide. Equally probable is that differences in the intensity of the light treatment used to evoke the behavioral responses are critically different. Work done on the PAC_1 knockout mice indicates that the loss of the receptor impacts the magnitude of light-induced phase delay and that this effect is seen primarily under low light intensities (Hannibal *et al.*, 2008). The light treatments that we used in the present study are sub-saturating in terms of evoking a behavioral response (Colwell *et al.*, 2004), while those used by the Gillette group may have been saturating (Beaulé *et al.*, 2009). While not uncommon in the field of behavioral genetics (e.g. Wahlsten *et al.*, 2003), these types of differences indicate the need for caution in the interpretation of the results from these different models. Despite these differences, it is worth emphasizing that all of these studies consistently indicate that PACAP is important in the photic regulation of the circadian system and suggest a role for PACAP as a modulator of the effects of light on the circadian system.

In the present study, we found that VIP-deficient mice were less responsive to light in the night as measured by the photic induction of FOS expression and phosphorylation of MAPK within the SCN. We found that the magnitude of light-induction of FOS was reduced in both the early and late night. Previous work with the *Vipr2* −/− mice reported that light at ZT12.5 caused a minimal FOS induction in the SCN (Harmar *et al.*, 2002) compared with WT. A later study with these same mice found that the *Vipr2* −/− mice still had a significant induction of FOS in the night (Hughes *et al.*, 2004). These later results are consistent with the present data and suggest that VIP/$VPAC_2R$ signaling is critical for normal magnitude of light-induction of gene expression in the SCN.

Photic activation of the clock gene, *Per1*, is likely to be critical for the entrainment of the molecular clock within SCN neurons (e.g.



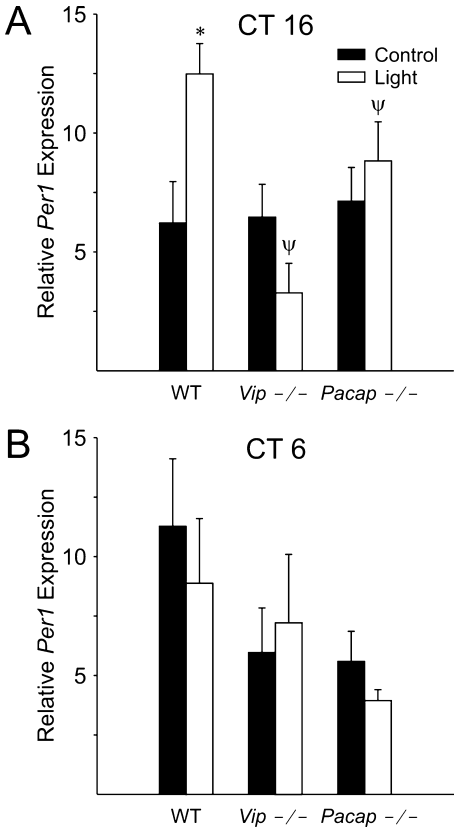


FIG. 5. Loss of vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) alters the photic regulation of *Period1* (*Per1*) expression in the adrenals. Mice were held in DD and wheel-running activity was measured to determine circadian phase. Mice were exposed to light (white light, 1.5×10^{-1} uW/cm^2; 10 min duration) in the subjective night (circadian time; CT16) or day (CT6), and tissue was collected 60 min after the beginning of the light treatment. Tissue from time-matched controls was collected for comparison. Quantitative real-time PCR was used to measure *Per1* mRNA in the adrenals (n = 3–4 per group). Expression levels of *Per1* were normalized to *Actb*. (A) Wild-type (WT), but not VIP- or PACAP-deficient, mice displayed significant increases in the levels of *Per1* in the adrenals after exposure to light at CT16 when compared with untreated controls. The magnitude of *Per1* expression in the light-treated groups was significantly reduced in the VIP- and PACAP-deficient mice when compared with WT mice. There were no significant differences between the baseline levels of *Per1* in the adrenals at CT16. (B) At CT6, light exposure did not alter *Per1* levels in any of the genotypes. See Fig. 2 legend for symbol definitions.

Shigeyoshi *et al.*, 1997; Akiyama *et al.*, 1999; Wakamatsu *et al.*, 2001; Masubuchi *et al.*, 2005). We found that the PACAP-deficient mice did not exhibit a reduction in the magnitude of the light-induction of *Per1* compared with WT controls. In the early night (CT16), the light-evoked increase in *Per1* was actually larger in the PACAP-deficient mice compared with WT. Previous work has reported that PAC_1R-deficient mice exhibit a reduction in the magnitude of light-induction of *Per1* when measured in the early night but not the late night (Hannibal *et al.*, 2001). Conversely, microinjection of PACAP into the lateral ventricle during early night (CT16) caused a moderate induction of *Per1* in the mouse SCN *in vivo* (Minami *et al.*, 2002), and application of PACAP causes an induction of *Per1* in the rat SCN *in vitro* (Nielsen *et al.*, 2001). We found that the VIP-deficient mice exhibited a significant reduction in the magnitude of the light-induction of *Per1* message compared with WT controls at CT16, while at CT23 levels of light-evoked *Per1* were reduced but not to a significant degree. In the *Vipr2* −/− mice, the light-induction of PER1 was reported to be completely eliminated in the SCN (Harmar *et al.*, 2002). Application of VIP causes an induction of *Per1* in the rat SCN *in vitro* (Nielsen *et al.*, 2002). This observation raises the possibility that VIP can mediate, not just modulate, the light-induction of gene expression in the SCN. However, our results are more consistent with VIP having a modulatory role, i.e. light still induces FOS and *Per1* within the SCN of VIP-deficient mice but the magnitude of the response is reduced during the night.

Light-regulated gene expression in adrenals

Light also regulates the expression of clock genes in peripheral organs through a mechanism that is dependent upon the SCN (Ishida *et al.*, 2005). Anatomical studies have provided evidence that the paraventricular nucleus (PVN) receives VIP-positive innervations from the SCN (Mezey & Kiss, 1985; Teclemariam-Mesbah *et al.*, 1997; Kallo *et al.*, 2004). The PVN neurons send projections throughout the CNS and endocrine system, providing multiple pathways by which the SCN can convey temporal information to the brain and body, with the autonomic nervous system and the hypothalamic-pituitary-adrenal axis thought to be critical outputs (e.g. Swanson *et al.*, 1983; Buijs *et al.*, 1999; Kalsbeek *et al.*, 2006). Furthermore, transneuronal and retrograde viral tracing shows multi-synaptic pathways between the SCN and the adrenal glands (Buijs *et al.*, 1999, 2003). VIP, PACAP and their receptors are also expressed in the adrenal glands, and could well integrate downstream signals from the SCN (Shiotani *et al.*, 1995; Shioda *et al.*, 2000; Harmar *et al.*, 2004). In the present study, we were able to demonstrate a robust light-induction of *Per1* in the adrenals in the subjective night (CT16). The loss of PACAP and VIP reduced the magnitude of the light-evoked increase in *Per1* levels compared with WT controls. This is consistent with the finding that the SCN is necessary for light-induced upregulation of *Per1* expression

FIG. 4. Loss of vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) alters the photic regulation of *Per1* expression in the SCN. Mice were held in DD and wheel-running activity was measured to determine circadian phase. Experimental mice were then exposed to light (see above) at one of three phases of the daily cycle, and tissue collected 60 min after the beginning of the light treatment. Tissue from time-matched control mice was also collected for comparison. ISH was used to measure *Per1* expression in the SCN (n = 4–5 per group). (A) Photomicrographs of autoradiograms of the SCN from VIP-deficient, PACAP-deficient and C57 littermates (wild-type; WT) mice exposed to light at CT23 along with time-matched controls. (B) All three genotypes displayed significant increases in the number of *Per1* message in the SCN after exposure to light at CT23 when compared with untreated controls. The magnitude of this light-induction was not significantly reduced in either VIP- or PACAP-deficient mice when compared with WT mice. There were no significant differences between the baseline levels of *Per1* in the SCN at CT23. (C) Photomicrographs of the SCN from WT, VIP −/− and PACAP −/− mice exposed to light at circadian time (CT)16 along with time-matched controls. (D) All three genotypes displayed significant increases in the number of *Per1* message in the SCN after exposure to light at CT16 when compared with untreated controls. The magnitude of this light-induction was significantly reduced in VIP- and enhanced in PACAP-deficient mice when compared with WT mice. There were no significant differences between the baseline levels of *Per1* in the SCN at CT16. (E) Photomicrographs of the SCN from VIP −/−, PACAP −/− and WT mice exposed to light at CT6 along with time-matched controls. (F) VIP-deficient, but not WT or PACAP-deficient, mice displayed significant increases in the levels of *Per1* in the SCN after exposure to light at CT6 when compared with untreated controls. However, the magnitude of *Per1* expression in the light-treated groups did not vary between the genotypes. Baseline levels of *Per1* in the SCN at CT6 were significantly reduced in the VIP-deficient mice and significantly increased in PACAP-deficient mice when compared with WT mice. There were significant differences in the light-evoked fold-increase in the levels of *Per1* in the SCN between the genotypes (WT: 1.0 ± 0.7, n = 4; VIP: 1.8 ± 0.3, n = 4; PACAP 0.85 ± 0.03, n = 3). See Fig. 2 legend for symbol definitions.

(Ishida *et al.*, 2005). The finding that the loss of PACAP interferes with the light-induction of *Per1* in the adrenals, but not the SCN, is of interest. This finding provides a clear indication that these peptides can have a different regulatory role in the photic regulation of clock gene expression in the periphery than in the central clock. The adrenal gland has two anatomically and functionally different components: the cortex and the medulla. Previous work using ISH indicates that the light-induced changes in *Per1* were mostly in the cortical region of the adrenals (Ishida *et al.*, 2005), and we can assume that the photic regulation that we observe is also from this region. Given our current understanding of the circadian system as a multi-oscillator control system, understanding the mechanisms by which light regulates gene expression in the periphery is an important area for future work.

Summary

Anatomical evidence has demonstrated that PACAP is expressed in a subset of retinal ganglion cells that terminate on the ventral portion of the SCN (Hannibal & Fahrenkrug, 2004), whereas VIP is expressed in a subset of SCN neurons within the ventral SCN (Antle & Silver, 2005; Morin *et al.*, 2006) that then send projections throughout the nucleus. In this study, we used mutant mice deficient in either PACAP or VIP to explore the role of these closely-related peptides in the light-induced gene expression within the SCN. We found that the loss of PACAP reduced the magnitude of light-induced gene expression within the SCN: FOS and pMAPK were both impacted. Our data are consistent with the broader literature that suggests a role for PACAP and PAC1 receptors in amplifying the magnitude of light-induced gene expression within the SCN as a modulator of glutamatergic signaling and the resulting calcium influx. The present literature is not consistent concerning the relative role for PACAP/PAC1 signaling in modulating light-induced phase delays and advances, with clear differences emerging between different genetic models. We also found that the loss of VIP reduced the magnitude of light-evoked gene expression within the SCN during the subjective night: FOS, p-MAPK and *Per1* were all impacted. Given the anatomical localization of VIP in the retino-recipient SCN neurons, the simplest explanation is that the loss of VIP prevents the spread of photic information from the ventral neurons to the rest of the SCN circuit. While not a focus of the present study, our data are consistent with this simple model in that the photic induction of gene expression in the dorsal SCN appears to be particularly sensitive to the loss of VIP. Finally, VIP-deficient mice also exhibited light-induced gene expression, but not behavioral responses, during the subjective day. During this phase, the baseline levels of FOS, p-MAPK and *Per1* were all reduced in the SCN of VIP-deficient mice compared with WT mice. Thus, our data are consistent with the broader literature that suggests a role for VIP and $VPAC_2R$ in the transmission of light information within the SCN and perhaps in the 'gating' of the circadian system. Together, our data demonstrate a clear role for PACAP and VIP peptides in the photic regulation of gene expression within the SCN, and point out the need for additional studies to understand the underlying mechanisms.

Acknowledgements

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Abbreviations

CT, circadian time; DD, constant dark; DTT, dithiothreitol; IHC, immunohistochemistry; ISH, *in situ* hybridization; LD, light–dark; NMDA, *N*-methyl-D-aspartate; PACAP, pituitary adenylate cyclase-activating polypeptide; PBS, phosphate-buffered saline; *Per1, Period1;* p-MAPK, phosphorylated mitogen-activated protein kinase; PVN, paraventricular nucleus; RHT, retinohypothalamic tract; RT-PCR, reverse transcriptase polymerase chain reaction; SCN, suprachiasmatic nucleus; SDS, sodium dodecyl sulfate; SSC, standard sodium citrate; VIP, vasoactive intestinal peptide; WT, wild-type.

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