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Selective deficits in the circadian light response in mice lacking PACAP

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Colwell, C. S., S. Michel, J. Itri, W. Rodriguez, J. Tam, V. Lelièvre, Z. Hu, and J. A. Waschek. Selective deficits in the circadian light response in mice lacking PACAP. *Am J Physiol Regul Integr Comp Physiol* 287: R1194–R1201, 2004. First published June 24, 2004; doi:10.1152/ajpregu.00268.2004.—Previous studies indicate that light information reaches the suprachiasmatic nucleus through a subpopulation of retinal ganglion cells that contain both glutamate and pituitary adenylyl cyclase-activating peptide (PACAP). Although the role of glutamate in this pathway has been well studied, the involvement of PACAP and its receptors is only beginning to be understood. To investigate the functions of PACAP in vivo, we developed a mouse model in which the gene coding for PACAP was disrupted by targeted homologous recombination. RIA was used to confirm a lack of detectable PACAP protein in these mice. PACAP-deficient mice exhibited significant impairment in the magnitude of the response to brief light exposures with both light-induced phase delays and advances of the circadian system impacted. This mutation equally impacted phase shifts induced by bright and dim light exposure. Despite these effects on phase shifting, the loss of PACAP had only limited effects on the generation of circadian oscillations, as measured by rhythms in wheel-running activity. Unlike melanopsin-deficient mice, the mice lacking PACAP exhibited no loss of function in the direct light-induced inhibition of locomotor activity, i.e., masking. Finally, the PACAP-deficient mice exhibited normal phase shifts in response to exposure to discrete dark treatments. The results reported here show that the loss of PACAP produced selective deficits in the light response of the circadian system.

dark pulses; entrainment; masking; pituitary adenylyl cyclase-activating peptide; suprachiasmatic nucleus

IN MAMMALS, THE NEURAL STRUCTURE responsible for most circadian behaviors can be localized to a bilaterally paired structure in the hypothalamus, the suprachiasmatic nucleus (SCN). Although previous studies suggest that each SCN neuron may be an independent oscillator (18), these cells must be synchronized to each other and to the environment to function adaptively. This process of entrainment is thought to occur through daily light-induced phase advances and delays of the endogenous clock. Most of the features of the entrainment to a full light-dark (LD) cycle can be mimicked by exposing an organism to one or two brief pulses of light per daily cycle. The response of the circadian system to brief light pulses is phase dependent such that the same light treatment can either phase delay (early subjective night), phase advance (late subjective night), or have no effect on the circadian oscillator (subjective day). A great deal of the research in understanding the photic entrainment of the circadian system has focused on understanding the functional response of the system to brief light pulses and the underlying cellular/molecular mechanisms. The SCN receives photic information directly through a monosynaptic projection from the retina known as the retinal hypothalamic tract (RHT). The RHT comprises a distinct subset of retinal ganglion cells that recent studies demonstrate contain a novel photopigment melanopsin (Opn4) and are directly light sensitive (3, 17). These ganglion cells appear to express and utilize the neuropeptide pituitary adenylyl cyclase-activating peptide (PACAP) and glutamate as cotransmitters to communicate with the SCN.

Although PACAP-like immunoreactivity is localized in terminals of neurons of the retinal ganglion cells innervating the SCN (11, 13), the closely related peptide vasoactive intestinal peptide (VIP) is expressed in ventrolateral SCN neurons. Two high-affinity receptors for PACAP are expressed in the SCN (4, 5, 32). One is relatively selective for PACAP (PAC1), and the other is selective for both VIP and PACAP (VPAC2; see Ref. 14). Because PACAP can potentially act on both of these receptors in the SCN, and because VIP is also highly expressed, it is very important to dissect the role of each ligand and receptor separately. Circadian regulation has also been examined in mice deficient in VIP (7), VPAC2 (15), and PAC1 (12).

A simple prediction is that PACAP functions as a transmitter linking the light-sensitive retinal ganglion cells with the SCN and that the loss of PACAP or its receptors should attenuate light-induced phase shifts of the circadian system. Previous studies have found some support for this simple model. Microinjections of PACAP in the SCN region in vivo can cause phase shifts (2, 16, 22, 29), and administration of a PACAP receptor antagonist or an antibody against PACAP attenuates light-induced phase delays (2). A previous study found that PACAP-deficient mice exhibited attenuated light-induced phase shifts, but this was only significant with light exposure in the late night (19). However, there is another set of data that suggest that PACAP may produce very different effects on light-induced phase advances. For example, the administration of anti-PACAP antibodies in the cerebral ventricles of hamsters enhanced light-induced phase advances (6). Similarly, the circadian system of mice deficient in the PAC1 receptor (12) exhibited an attenuation of light-induced phase delays when exposed to light in the early subjective night but exhibited light-induced phase delays, rather than advances, when exposed to light in the late subjective night. Given some of the inconsistencies in this previous data, we sought to test the

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hypothesis that the loss of PACAP would attenuate the light response of the circadian mammalian system through the generation and behavioral analysis of a new line of PACAP-deficient mice.

METHODS

Establishment of PACAP-deficient mice. We generated mice with specific mutations in the PACAP gene using an embryonic stem (ES) cell-based approach. First, a bacteria artificial chromosome (BAC) library from strain 129 mice was screened with a mouse PACAP cDNA (33). Three independent genomic BACs were obtained. Several restriction fragments that hybridized to the cDNA were subcloned into Bluescript. Using exon-specific probes, a map of the mouse PACAP gene was constructed (Fig. 1*A*). PACAP, encoded at the 5′-end of exon 5, and PACAP-related peptide (PRP), encoded on exon 4 as well as exon 3, were excluded in the targeting construct and replaced with a neomycin cassette (Fig. 1*A*, *middle*). PRP is a PACAP-like sequence on the PACAP peptide precursor that is flanked by typical neuropeptide cleavage motifs and thus is a putative neuropeptide. Although we cannot rule out an action by PRP, this peptide has no known function within the nervous system. The Herpes simplex thymidine kinase gene was placed at the 3′-end to enable both positive and negative antibiotic selection. This construct was introduced into ES cells (129/sv agouti, wild-type albino locus) by the University of California Los Angeles (UCLA) transgenic/ES cell core facility. To screen clones for homologous recombination, Southern blots were prepared containing genomic DNA digested with *Bam*HI. These were probed with a *Bam*HI/*Kpn*I fragment that consisted of gene sequences entirely outside of the targeting vector (the location of the probe is shown in Fig. 1*A*). The probe could thus hybridize only to endogenous gene sequences (wild type and recombinant), and not to sequences on the targeting vector that could be present because of random incorporation of the injected DNA fragment in the genome. This analysis identified three different ES clones that contained the gene mutated by homologous recombination. These were injected in blastocysts of C57BL/6 mice. Several chimeric mice were obtained from each of the three clones. Using the same probe and Southern analysis procedure used for screening ES cell clones, germ line transmission was confirmed in offspring of chimeric mice derived from two of these clones (Fig. 1*B*). The ratio of PACAP(−/−) mice (backcrossed), determined at the time of genotyping, was significantly lower than that expected according to Mendelian rules (we observe 50–80% of the expected number of nulls). This suggests some lethality of null animals, either in utero or postnatally before genotyping. However, the survivors do not exhibit significant morbidity or mortality in the normal housing conditions.

PACAP RIA. Hypothalamus, cerebral cortex, kidney, and stomach were removed from PACAP(+/+) and PACAP(−/−) animals (3 mice/group) and weighed. Tissues were boiled in 1.5 ml distilled water for 20 min, homogenized using a Polytron ultraturax, and spun for 10 min at 1,500 relative centrifugal force (RCF). Supernatants were removed and set aside on ice. Pellets were reconstituted in acetic acid (0.5 M) and centrifuged for 10 min at 1,500 RCF. Supernatants were combined and centrifuged one more time for 10 min at 1,000 RCF. Supernatants were transferred to new tubes. Of this, 500-μl portions were transferred to tubes, dried using a SpeedVac, and reconstituted in 0.5 M acetate buffer. Aliquots were diluted in triplicates before RIA analysis using a kit from Phoenix Pharma-

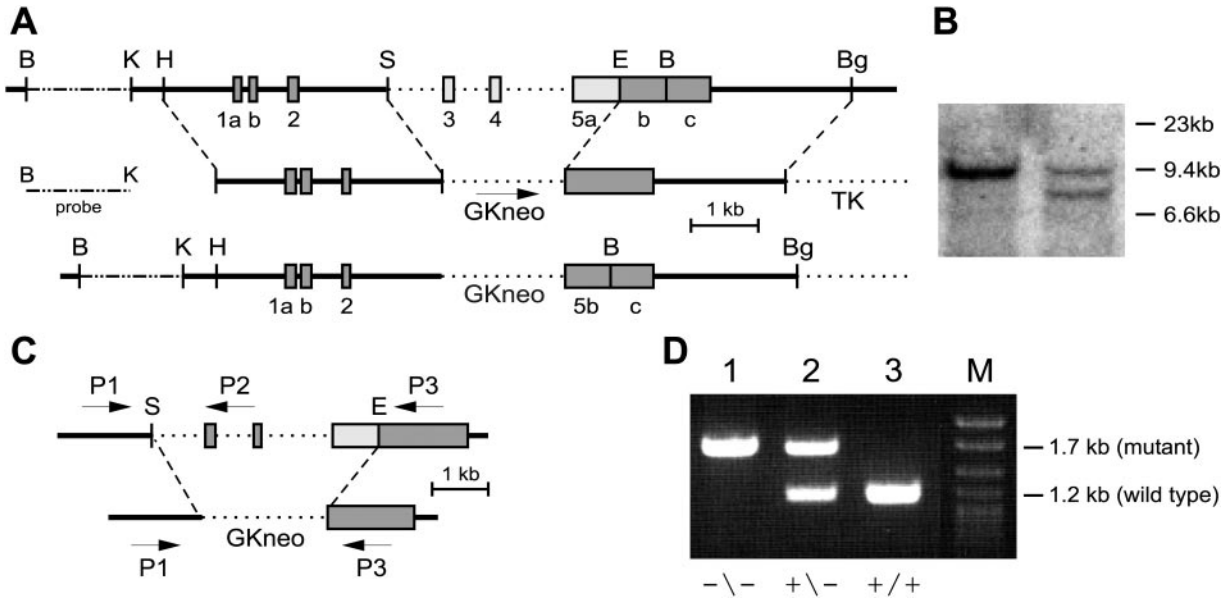

Fig. 1. PACAP gene knockout strategy and conformation of germ line transmission. *A*: the *top segment* is the wild-type PACAP gene, with exons (*1–5*) indicated by boxes and introns between them indicated by lines. The light gray colored part of the gene (containing all PACAP encoding sequences) has been removed in the targeting construct (*middle segment*) and human 3-phosphoglycerate kinase promoter/neomycin cassette (GKneo) added. The *bottom segment* represents the predicted structure of the gene altered by homologous recombination. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Spe*I; TK, thymidine kinase; X, *Xho*I. *B*: disruption of the endogenous PACAP gene in offspring of a chimeric mouse made with ES cells. Shown are tail DNA samples from 2 offspring, digested with *Bam*HI and analyzed by Southern blot. The blot was probed with the 5′ 1.4-kb *Bam*HI/*Kpn*I fragment shown in *A*. *Lane 2* is from a mouse heterozygous for the mutated gene, and it contains bands at the predicted sizes for the endogenous and mutated gene (9.0 and 7.7 kb, respectively). *C* and *D*: PCR analysis of PACAP +/+, +/−, and −/− mice. Relevant portions of the wild-type (*top*) and mutated PACAP (*bottom*) genes are shown in *C*. The arrowheads indicate the positions of the 3 primers used in the PCR reaction. *D* shows an ethidium-stain agarose gel of reaction products from 2 offspring alongside a size marker (M). As indicated at *right*, the mutant allele gives a 1.7-kb band (generated by primers P1 and P3), whereas the wild-type allele gives a product of 1.2 kb, generated by P1 and P2. No band is amplified from the wild-type allele using P1 and P3 primers because the predicted 3.1-kb-sized band is not efficiently amplified under these PCR conditions.

ceuticals (Belmont, CA), and radioactivity was measured with a gamma counter (Bechman Coulter, Fullerton, CA).

Behavioral studies. To obtain mice homozygous for the PACAP gene mutation, heterozygous male offspring of chimeric mice were interbred with C57BL/6 females. Initial litters obtained were thus of mixed C57BL/6 × 129/sv background. Behavioral studies utilized mice that had been backcrossed for at least six generations into a C57BL/6 background. Homozygous PACAP mutant mice and PACAP(+/+) controls from the same litter were analyzed in most cases, but, occasionally, when a PACAP(+/+) male littermate was unavailable, an age-matched control mouse from another litter of the same genetic background was used. In all studies, the 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.

Male mice, at least 8 wk of age, were housed individually, and their wheel-running activity was recorded as revolutions (rev)/3-min interval. The running wheels and data acquisition system were obtained from Mini Mitter (Bend, OR). The animals were exposed to a 12:12-h LD cycle (light intensity 300 lux) for 2 wk. Next, the animals were placed in constant darkness (DD) to assess their free-running activity pattern. Some mice in DD were exposed to a brief light treatment at circadian time (CT) 16 or 22 (CT 12 is defined by the locomotor activity onset). After each treatment, the animals were allowed to free-run undisturbed in DD for 14 days. The light stimulus that was used to induce phase shifts was an exposure to white light (500 or 50 lux) for 10 min. Phase shifts in the activity rhythm were determined by measuring the phase difference between eye-fitted lines connecting the onset of activity for a period of 7 days before and 10 days after an experimental manipulation. Measurements were made by investigators "blind" to the experimental group. To estimate the steady-state phase shifts produced, one day of data after treatments that cause phase delays and four days of data after treatments that cause phase advances were excluded from the analysis. Other mice in DD were also exposed to a light treatment (white light, 60 min, various intensities) during their activity period to measure negative masking behavior (i.e., light-induced suppression of activity). The number of revolutions during this light treatment was compared with the number recorded during the same hour on the previous day in the dark (23). Some mice were maintained in constant light (LL) conditions (50 lux) for 3–4 wk. Stimulus intensity (lux) was measured with a light meter (BK Precision, Yorba Linda, CA). All handling of animals was carried out either in the light portion of the LD cycle or in DD with the aid of an infrared viewer (Industrial Technologies, St. Petersburg, Russia).

The locomotor activity rhythms of mice were analyzed by periodogram analysis combined with the χ^2-test with a 0.1% significance level (El Temps, Barcelona, Spain) on the raw data. The periodogram shows the amplitude (= power) of periodicities in the time series for all periods of interest (between 20 and 31 h in 3-min steps). The power values were normalized to the percentage of variance derived from the Qp values of the periodogram (Qp × 100/N; N = total no. of data points) according to the calculated 0.1% significance level, where Q_p is a ratio of the variance at a period over the variance of the mean. During DD, we found that a power value >30% indicated a strong and coherent activity rhythm. Slopes of an eye-fitted line through the onsets were also used to confirm period estimates made with the periodogram analysis. To estimate the cycle-to-cycle variability in activity onset, a linear regression to 15 cycles of onset of activity was calculated. The onset of activity for each cycle was defined as the phase at which the animal's activity levels first equaled the mean activity level for that cycle. The divergence between the measured and predicted onsets by linear regression was then determined, and the average difference was calculated for each animal. The duration of each cycle devoted to running-wheel activity is designated alpha (α), whereas the duration of rest is designated rho (ρ). To measure these parameters, the average pattern of activity (i.e., the form estimate) was determined at moduloperiod for each animal in DD for 15 cycles. Next, for each waveform, α was calculated as the time during which the motor activity was above the median.

Statistical analyses. Between-group differences were typically evaluated using *t*-tests or Mann-Whitney rank sum tests when appropriate. Two-way ANOVA was used to test the possibility that light-induced suppression of activity varied between the two genotypes. Values were considered significantly different at $P < 0.05$. All tests were performed using SigmaStat (SPSS, Chicago, IL). In the text, values are shown as means ± SE.

RESULTS

Generation and behavioral analysis of PACAP-deficient mice. To test the hypothesis that PACAP is required for photic regulation of the circadian system, the PACAP gene was disrupted by removal of PACAP-encoding sequences on the upstream exon (Fig. 1*A*). Southern blot analysis revealed the occurrence of successful recombination events in an ES cell clone and germ line transmission from this clone (representative analysis showing germ line transmission shown in Fig. 1*B*). Subsequently, a PCR strategy was developed to identify wild-type, heterozygous, and null PACAP gene mutants generated from heterozygous crosses (Fig. 1, *C* and *D*). RIA confirmed a loss of PACAP in mice homozygous for the mutation (Fig. 2).

Behavioral analysis of the circadian rhythm in wheel running activity was carried out using mice [PACAP(+/+), PACAP(−/−)] on the C57BL/6 background. In these experiments, PACAP(−/−) and PACAP(+/+) littermate mice were individually placed in a cage containing a running wheel, and their daily activity was recorded. The animals were first synchronized for 2 wk to an LD cycle (12:12). Under these conditions, both groups synchronized to the LD cycle and exhibited diurnal rhythms in activity (Fig. 3). There was no significant between-group difference in the phase of activity onset or in cycle-to-cycle variability in activity onset. Wild-type mice and the PACAP(−/−) mutants entrained normally and consolidated their wheel-running activity to the dark period of the LD cycle. There was no significant difference in the amount of activity observed during the light portion of the LD cycle between the genotypes. We examined the ability of these mice to resynchronize to new LD cycles that were advanced or

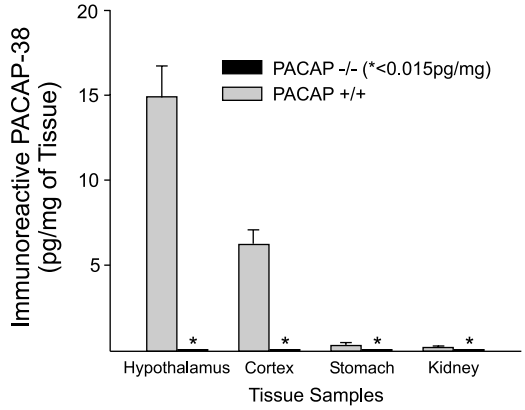

Fig. 2. RIA for PACAP. Tissue homogenates from various regions were sampled from PACAP(−/−) (n = 3) and PACAP(+/+) littermates (n = 3). Levels of PACAP-38 were below the limits of detection (0.015 pg/mg) in all regions sampled, including the hypothalamus of PACAP(−/−) mice.

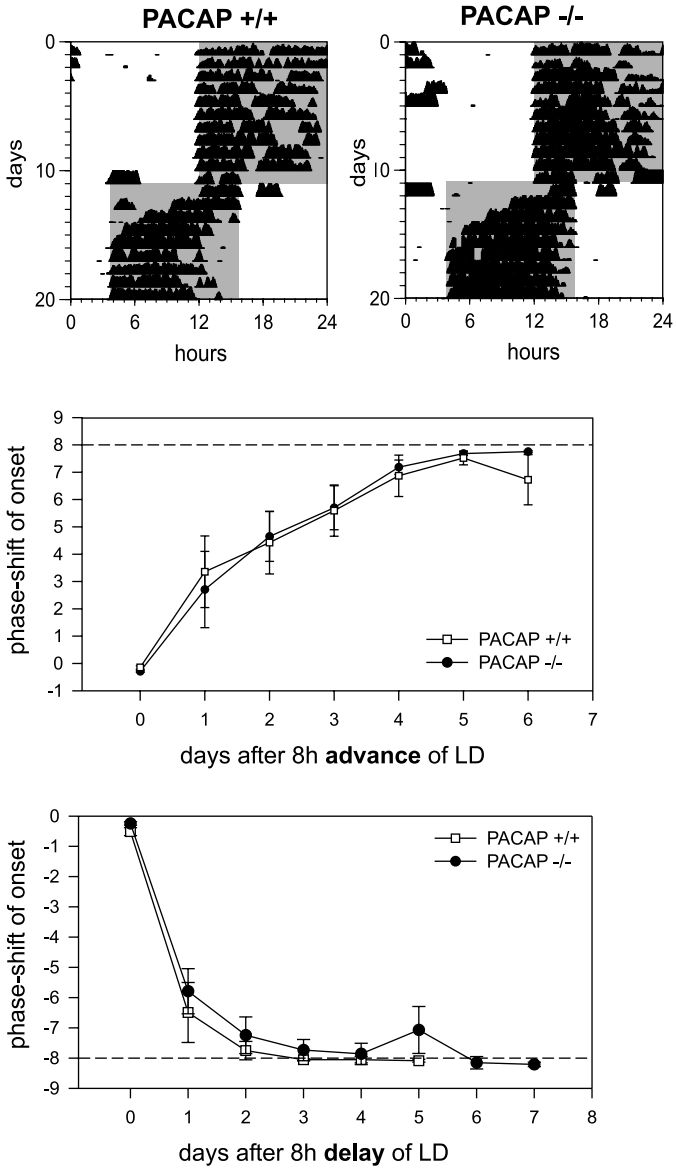

Fig. 3. Resynchronization to new light-dark (LD) cycles was not impacted by the loss of PACAP. Examples of wheel-running activity records from PACAP(+/+) and PACAP(−/−) mice. Animals were initially entrained to an LD cycle (12:12 h) and then subjected to an 8-h shift in the phase of light onset. Each horizontal row represents an activity record for a 24-h day. Successive days are plotted from *top* to *bottom*. *Top*: examples of the transients that occur during the reentrainment to the new LD cycle. *Middle*: daily rate of reentrainment to light-induced phase advance. *Bottom*: daily rate of reentrainment to light-induced phase delays. PACAP-deficient mice (n = 8) exhibited reentrainment that was not significantly different from littermate controls (n = 7).

delayed by 8 h. For both strains of mice, advances are accomplished over five cycles, whereas delays occur rapidly and are completed within three cycles. There was no difference ($P > 0.05$) between PACAP(−/−) and -(+/+) mice in the rate of reentrainment to the new LD cycle measured at each transient cycle (Fig. 3). In the LD condition, the only significant effect that we observed was that the mean wheel-running activity levels were significantly higher in the PACAP(−/−) animals [PACAP(−/−): 448 ± 37 rev/h, $P < 0.01$, n = 13; PACAP(+/+): 286 ± 29 rev/h, n = 11]. However, it is important to note that, in an LD cycle, photic input organizes the temporal pattern of activity by synchronizing an endogenous clock to the period of the environmental signal (entrainment) and directly regulating activity (masking). To distinguish between these two effects of lights, it is necessary to place the mice in DD and measure their activity rhythms in the absence of photic cues.

Behavioral rhythms in DD. First, to determine whether the loss of PACAP had an impact on the generation of circadian rhythms in wheel-running activity, the free-running activity rhythms were measured in PACAP-deficient mice and littermate controls in DD. In general, compared with littermate controls, the PACAP(−/−) mice exhibited several significant differences in circadian parameters; however, these differences were small in magnitude (Table 1). For example, the free-running period (Tau) of the PACAP(−/−) animals was significantly shorter than controls, but the difference was ~20 min. Perhaps because of this difference in period, the PACAP-deficient mice also exhibited a significant shortening of the phase angle of entrainment (ψ; Table 1). However, once released in DD, the activity of both the PACAP(−/−) and -(+/+) mice started from the phase predicted by the prior LD cycle. This suggests that the circadian rhythm in locomotor activity was synchronized to the LD cycle in both genotypes. Finally, a detailed analysis of the first 15 cycles in DD found small or no differences between the two genotypes in the coherence, α/ρ, and cycle-to-cycle variability in the activity rhythms (Table 1). These similarities were also reflected in the lack of difference in the power of periodicity in the time series analysis of the activity rhythms expressed by the mutant animals (Table 1).

Light-induced phase shifts of locomotor activity rhythms. To assess the effects of light on the circadian system, the phase shifts induced by single, discrete light treatments were examined (Fig. 4). Although PACAP(+/+) littermates exposed to bright, white light at CT 16 (500 lux for 10 min) showed a 118 ± 6 min (n = 12) phase delay, PACAP(−/−) mice had a 60% reduction in magnitude of phase delay after the same light treatment (47 ± 11 min, n = 8; $P < 0.001$). Similarly, PACAP(−/−) littermates exposed to light at CT 23 showed 49% reduction in magnitude of phase advance compared with controls [PACAP(−/−): 23 ± 4 min, n = 8; PACAP(+/+): 45 ± 6 min, n = 7, $P < 0.05$]. To look at the possibility the loss of PACAP may only impact the phase-shifting effects of bright light, neutral density filters were used to lower the light intensity to 50 lux (Fig. 5). Whereas PACAP(+/+) littermates

Table 1. *Comparison of circadian rhythm parameters of between PACAP (+/+) and PACAP (−/−) mice*

	PACAP (+/+) (n = 11)	PACAP (−/−) (n = 13)	Significance
Activity in DD, rev/h	390±37	485±40	$P<0.005$
Tau, h	23.71±0.06	23.3±0.09	$P<0.005$
ψ, min	−9.27±3.52	9.42±4.53	$P<0.005$
Precision, min	7.9±0.6	10.5±0.77	$P<0.005$
α, h	10.38±0.52	11.14±0.31	NS
Activity in α, %	92.17±1.68	96.13±0.63	$P<0.05$
α/ρ ratio	0.80±0.07	0.91±0.05	NS
Power, %variance	44.21±4.8	48.67±4.03	NS

Values are means ± SE; n, no. of mice. PACAP, pituitary adenyly cyclase-activating peptide; DD, constant darkness; rev, revolutions; α, duration of cycle devoted to running wheel activity; ρ, duration of rest. NS, not significant.

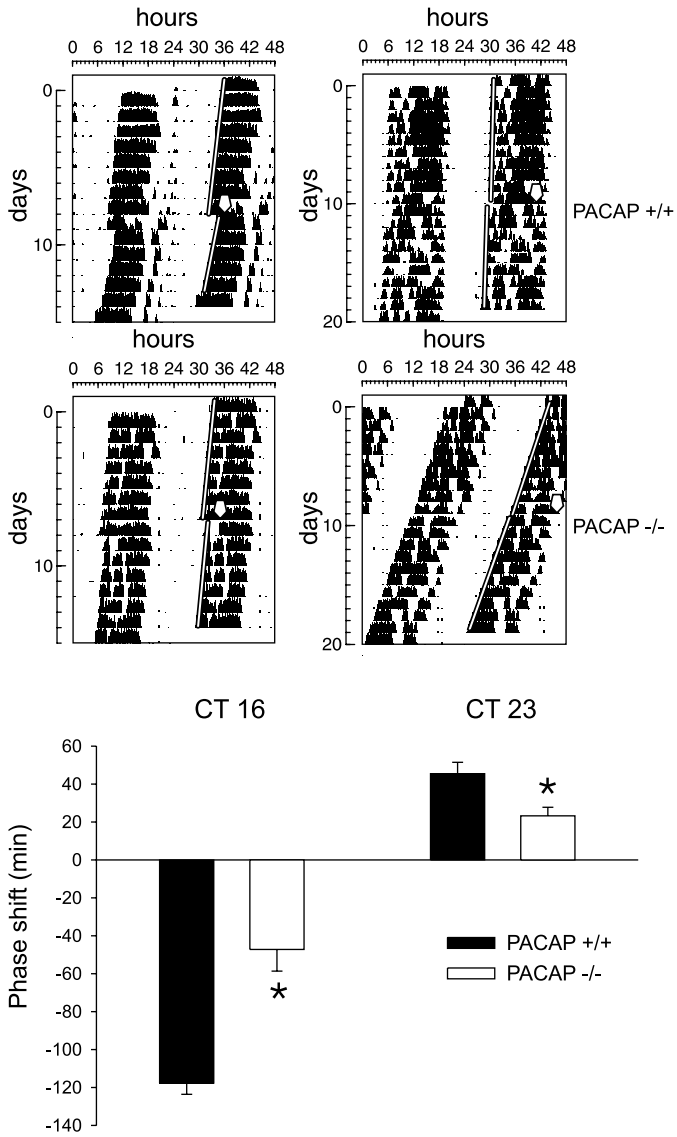

Fig. 4. Magnitude of light-induced phase shifts is reduced in PACAP-deficient mice. Examples of wheel-running activity records from PACAP(+/+) and PACAP(−/−) mice. Animals were initially entrained to an LD cycle (12:12) and then maintained in constant darkness (DD). All data are from DD. The time of the light treatment (500 lux, 10 min) is shown by the white arrow. The circadian system responds to brief light pulses with changes in the phase of activity onset. *Left*, light-induced delays that resulted from exposure to light at circadian time (CT) 16; *right*, light-induced phase advances that result from light exposure at CT 23. Histograms at *bottom* summarize the effects of light on light-induced phase delays and advances. At both phases, the PACAP-deficient mice exhibited a significant reduction in the effects of light treatment compared with PACAP(+/+) littermates. *Values that are significantly greater than those of the PACAP(+/+) controls ($P < 0.05$).

exposed to this dim light at CT 16 showed a 74 ± 10-min (n = 7) phase delay, PACAP(−/−) mice had a 67% reduction in magnitude of phase delay after the same light treatment (24 ± 8 min, n = 7; $P < 0.001$). Exposure to light at CT 6 did not cause phase shifts in either genotype (data not shown). Although mice always remained in their individual cages, the light exposure involved movement of the cage. Handling controls (n = 6/group) indicated that this movement did not cause phase shifts in either genotype at any of the three phases examined [handling at CT 6: PACAP(+/+): 11 ± 3 min, PACAP(−/−): 7 ± 3 min; handling at CT 16: PACAP(+/+): 0 ± 1 min, PACAP(−/−): 2 ± 1 min; handling at CT 23: PACAP(+/+): 6 ± 4 min, PACAP(−/−): 0 min].

Light-induced masking of locomotor activity. To investigate the role of PACAP in the direct light-induced suppression of locomotor activity, mice were exposed to 1 h of light of three different intensities during the dark portion of the LD cycle 2 h after lights off (Zeitgeber time (ZT) 14–15). The amount of activity (measured as rev/h) during this light treatment was compared with the number recorded during the same hour on the previous day in the dark. Three intensities of light (white light, 60 min) all acutely suppressed wheel running activity ("masked"), and these masking effects were similar [P = 0.125, F = 2.457, 2-way ANOVA in PACAP(−/−) and -(+/+) mice (Fig. 6)].

Effects of LL on period of locomotor activity and dark pulses. The next set of experiments was designed to investigate the role of PACAP in the photic modulation of the cycle length of the circadian oscillator. Under LL (50 lux) conditions, nocturnal rodents exhibit a lengthening of their free-running periods. Both wild-type and PACAP(−/−) mice exhibited wheel-running rhythm periods of >24 h in LL. As has been previously observed with melanopsin-deficient mice (27, 31), the PACAP(−/−) mice exhibited a slightly shorter period length in LL [PACAP(−/−): 24.6 ± 0.1 h, n = 13; PACAP(+/+): 24.9 ± 0.1 h, n = 13]. To test the hypothesis that PACAP mediates the effects of darkness on the circadian system, some of the mice in LL were exposed to 1 h of dark at CT 6 (Fig. 7). This treatment caused significant phase shifts in both strains of mice, and there was no difference ($P > 0.05$) in the magnitude of dark- and pulse-induced phase shifts [PACAP(+/+): 94 ± 9 min, n = 6; PACAP(−/−): 115 ± 8 min, n = 7]. Thus PACAP cannot mediate the effects of dark under these experimental conditions.

DISCUSSION

In the present study, we sought to better understand the role of PACAP by producing a new line of mice deficient in this neuropeptide and then characterizing the circadian phenotype

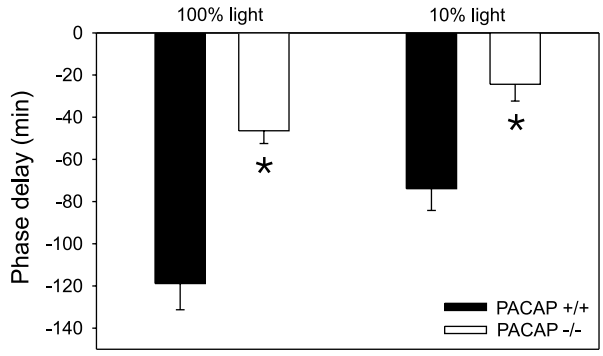

Fig. 5. Phase-shifting response to both bright and dim light was reduced in PACAP-deficient mice. Comparison of the magnitude of phase shifts to bright and dim light in PACAP(−/−) and PACAP(+/+) mice. Transgenic mice exhibited a 60% reduction in amplitude of phase delay in response to bright light (500 lux) and a 67% reduction in amplitude in response to dim light (50 lux). *Values that are significantly less than those of the controls ($P < 0.05$). Therefore, the loss of PACAP appears to diminish the response to both bright and dim light.

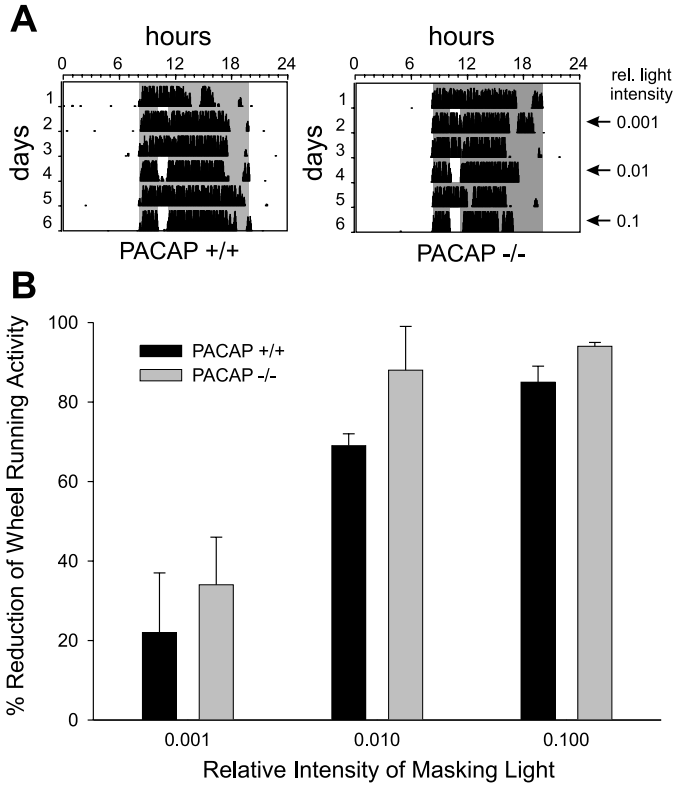

Fig. 6. Light-induced suppression of activity is not reduced in PACAP-deficient mice. Mice in an LD cycle were also exposed to a light treatment (white light, 60 min, 3 intensities) at ZT 14 to measure negative masking behavior, i.e., light-induced suppression of activity. *A*: examples of light-induced suppression of activity in PACAP(+/+) and PACAP(−/−) mice. *B*: histograms showing the mean suppression of activity for mice treated with three intensities of light. Relative intensities were generated by placing neutral-density filters in front of the light source. Therefore, for light treatments labeled "0.001," mice were exposed to a light that was 0.1% of the intensity at the source (50 lux). No. of revolutions during this light treatment was compared with the no. recorded during the same hour on the previous day in the dark, and the %reduction of activity was calculated. There were no significant differences between PACAP(−/−) and -(+/+) mice.

of these animals. Running-wheel activity was used exclusively as the marker of circadian output. The most striking deficit that we observed in the PACAP(−/−) mice was a significant reduction in the magnitude of the response to brief light exposure. This loss of function was seen with both dim and brighter light intensities. The loss of PACAP resulted in about a 50% reduction in the magnitude of both light-induced phase advances and phase delays. In an important way, these observations stand in contrast to the interpretations of several previous studies that have found evidence suggesting that PACAP may play dramatically different roles in the regulation of light-induced phase delays and advances. For example, the administration of anti-PACAP antibodies in the cerebral ventricles of hamsters was reported to enhance light-induced phase advances (6) and attenuate light-induced phase delays (2). Similarly, PAC1(−/−) mice exhibited larger phase delays in the early night and phase delays instead of phase advances in response to light in the late night (12). These studies have led to the proposal that the effects of PACAP on the light input to the circadian system are phase dependent in that PACAP will enhance light-induced phase delays but inhibit light-induced phase advances (6, 9).

In the present study, the most obvious consequence of the loss of PACAP is the altered magnitude in the response of the circadian system to light. We found a significant reduction in the magnitude of both light-induced delays (60% reduction) and advances (50% reduction) compared with littermate controls. Similar to our results, a recent study reported that PACAP-deficient mice exhibited phase delays that were reduced by ~30% and phase advances reduced by ~50% compared with wild-type controls (19). Interestingly, although the 30% reduction in phase delay was not statistically significant in that study, these PACAP-deficient mice also exhibited a significant reduction in the light induction of c-Fos in the SCN during the early night. There are many possible explanations for the differences in the magnitude of the deficit in light-induced phase delays between the two lines of PACAP-deficient mice. These include the different genetic background of the mice (Institute of Cancer Research strain vs. C57 BL/6 in our study) to the different light intensity and time point used to measure the phase delay (CT 15 vs. CT 16). In any case, we feel that the simplest explanation of the data is that PACAP is required for normal light-induced synchronization of the circadian system and plays a role modulating both light-induced advances and delays.

The loss of function that we observed in the PACAP-deficient mice appeared to be selective for the light input to the circadian system. We found little evidence that the loss of PACAP impacted the ability of the circadian system to generate circadian oscillations in constant conditions. There was a significant shortening of the period, but this frequency change

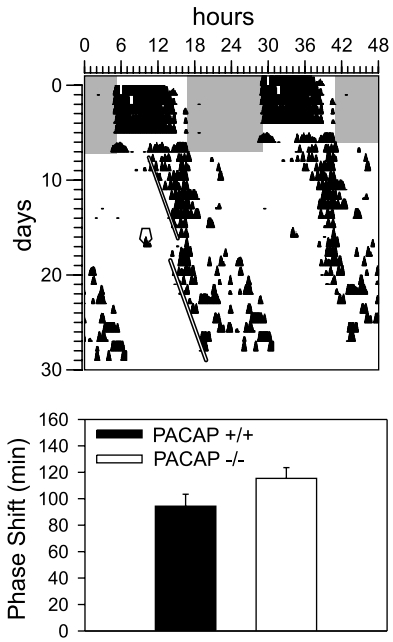

Fig. 7. Phase shifts induced by exposure to dark pulses were not affected by the loss of PACAP. Shown is an example of the wheel-running activity record of a PACAP(−/−) mouse. The animal was initially entrained to an LD cycle (12:12) and then maintained in LL for 4–6 wk. Gray shading represents the period of darkness. The mouse exhibits a free-running rhythm with a period longer than 24 h, as predicted by Aschoff's rules (1). The time of the dark treatment (60 min) is shown by the dark arrow. Exposure to dark at CT 6 resulted in a phase advance. There were no significant differences between dark- and pulse-induced phase shifts in PACAP(−/−) (n = 7) and PACAP(+/+) (n = 6) mice.

was quite small (20 min). This shortening of period may be an indirect consequence of the overall higher levels of running activity that we found in the PACAP-deficient mice (see Ref. 24). Overall, we see little evidence that the basic oscillatory feedback loop responsible for the generation of circadian rhythms was disrupted by the loss of PACAP and PRP. When organisms are held in LL, discrete dark treatments can effectively phase shift the circadian rhythm in wheel-running activity. Based largely on the finding that PACAP itself can cause phase shifts of circadian rhythms of neural activity recorded in the SCN, previous workers have speculated that PACAP may mediate the effects of arousal stimuli, such as dark pulses, that cause phase shifts during the daytime (10). In contrast, we found that the loss of PACAP did not interfere with the phase-shifting effects of dark exposure and, thus, PACAP does not mediate the effects of dark under these experimental conditions. Similarly, we found no impact of the mutation on the light-induced suppression of activity. This lack of effect is interesting given the observation that the loss of melanopsin impacts the masking effects of light (25). The melanopsin-containing retinal ganglion cells are likely to be utilizing both glutamate and PACAP to communicate light information to the SCN (11). Therefore, the role of PACAP may be selective for the entrainment function of the light input pathway, whereas glutamate may mediate both the masking and entraining functions of light. With normal masking, the PACAP-deficient mice appeared normal in a full LD cycle, and loss of function was only observed when measuring the response of the circadian system to discrete light pulses.

Physiological studies suggest that PACAP can modulate how SCN neurons respond to excitatory stimulation (8, 16, 20). At low concentrations, PACAP can enhance both *N*-methyl-D-glucamine (NMDA)-evoked currents in cultured hamster SCN neurons (16) and glutamate-induced Ca^{2+} transients in cultured rat SCN neurons (8, 20). By itself, PACAP has been shown to cause increases in cytosolic Ca^{2+} in cultured SCN neurons because of the release of Ca^{2+} from intracellular stores (8, 21). However, PACAP is not always excitatory; higher concentrations of this neuropeptide can inhibit NMDA-induced currents (16) and glutamate-induced phase shifts of electrical activity rhythms recorded in the SCN (6). Interestingly, in cultured SCN brain slices, application of nanomolar PACAP concentrations induced *mPer* expression by itself or, at higher micromolar concentrations, attenuated glutamate-induced mPeriod gene (mPer) expression (26). Certainly, a better understanding of the cellular mechanisms by which PACAP regulates SCN neurons is a critical step toward the development of an explanation for the phenotype observed in PACAP-deficient mice. Mechanistically, we speculate that PACAP functions to regulate the response of SCN neurons to glutamatergic stimulation and that loss of this modulation interferes with the synaptic signaling between the RHT and the SCN, resulting in a loss of sensitivity of the circadian system to photic stimulation. The data reported here clearly show that loss of PACAP is sufficient to produce selective disruptions in the synchronization of circadian oscillations to the environment.

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