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STRUCTURAL AND FUNCTIONAL CHANGES IN THE SUPRACHIASMATIC NUCLEUS FOLLOWING CHRONIC CIRCADIAN RHYTHM PERTURBATION

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Abstract—Circadian rhythms, generated in the suprachiasmatic nucleus (SCN), are synchronized to the ambient light/dark (LD) cycle. Long-term disruptions in circadian rhythms are associated with many health problems. However, the underlying mechanisms for such pathologies are not well understood. In the present study, we utilized a chronic jet lag paradigm consisting of weekly 6 h phase shifts in the LD cycle to investigate the circadian responses in behavior and in the functioning of the SCN following long-term circadian perturbation, and to explore the duration and direction dependent changes of the SCN using rats subjected to weekly phase advances or delays. Wheel-running activity was monitored over four weekly phase advances. The nocturnal activity pattern was re-established by the end of each shift, and the rate for recovering the nocturnality appeared to accelerate following multiple shifts. SCN function was assessed by the expressions of the protein product of clock gene PER1 and of two putative SCN output signals, arginine vasopressin (AVP) and prokineticin2 (Pk2). At the end of the 4th weekly advance, the amplitude of the PER1 rhythm in the SCN decreased, and this reduction was more prominent in the dorsomedial SCN than in the ventrolateral SCN. The levels of AVP and Pk2 expression were also attenuated in the SCN and in targets of its efferent projections. Comparing rats subjected to four or eight shifts of either delay or advance, the results revealed that the responses of the SCN depended on both duration and direction of the shifts, such that the level of PER1 expression further decreased at the end of the 8th compared to the 4th phase advance, but did not change significantly following phase delays. Taken together, the results suggest that rhythm perturbation could compromise the time-keeping function of the SCN, which could contribute to the associated health issues.

Key words: rat, circadian rhythm, suprachiasmatic nucleus, chronic jet lag, PER1.

The circadian system has major influence on the temporal organization of a vast array of behavioral and physiological functions, keeping internal processes synchronized with each other and entrained to cyclic features of the local environment (Klein et al., 1991). The circadian system has evolved the ability to synchronize or entrain to the stable and predictable light/dark (LD) conditions associated with the rotation of the planet (Pittendrigh, 1993). Thus, the environmental LD cycle is the most salient cue for entraining the circadian clocks in nearly all organisms (Aschoff, 1981).

In mammals, the principal circadian clock is located in the suprachiasmatic nucleus (SCN) (Klein et al., 1991). Environmental lighting cues received by the retina are conveyed to the SCN through the retinohypothalamic tract (Moore and Lenn, 1972). Neurons in the SCN are heterogeneous and are organized into at least two functionally distinct sub-regions, namely the retinorecipient core or ventrolateral (VL) region and the shell or dorsomedial (DM) region (Antle and Silver, 2005; Yan et al., 2007). Entrainment of the circadian clock of the SCN involves a series of molecular, cellular and intercellular events, such as the acute induction of clock genes, neuronal activation and the propagation of light signals from the retinorecipient VL region to the rest of the SCN through intercellular signaling pathways (Yan et al., 1999; Meijer and Schwartz, 2003; Antle et al., 2009). Clock genes that constitute the molecular clock exist not only in the SCN, but also in other brain regions, peripheral tissues/organs and even in cultured cell lines (Yoo et al., 2004; Schibler and Naef, 2005; Liu et al., 2007). These findings illustrate the hierarchical organization of the circadian system, in which the SCN sits at the top of the organizational hierarchy, sending output signals to the local clocks across the brain and body to produce coherent rhythmic behavioral and physiological responses (Davidson et al., 2003).

Disruptions in the temporal coordination of the circadian system, as seen in human shift workers and in animal models, compromise cognitive functions and long-term health (Penev et al., 1998; Abbott, 2003; Hastings et al., 2003; Davidson et al., 2006; Sahar and Sassone-Corsi, 2007; Martino et al., 2008). However, how the circadian system, and in particular the SCN, responds to chronic circadian disruptions is not well understood. The objective of the present study was to explore the neural mechanisms underlying long-term rhythm perturbation. The approach utilized a chronic jet-lag paradigm, in which the animals experience weekly 6 h phase shifts in their LD cycle. Previous studies using a single 6 h phase advance have revealed dissociated circadian oscillations in clock gene expression and neural activity of SCN neurons, and of

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Abbreviations: AVP, arginine vasopressin; DD, constant darkness; DM, dorsomedial; ICC, immunocytochemistry; ir, immunoreactive; LD, light/dark cycle; OD, optical density; PK2, prokineticin2; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; VL, ventrolateral; vSPVZ, ventral sub-paraventricular zone; ZT, Zeitgeber time.

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functionally distinct sub-regions of the nucleus (Nakamura et al., 2001; Reddy et al., 2002; Nagano et al., 2003; Davidson et al., 2009). Therefore, we predicted that multiple shifts would further dissociate oscillating components within the SCN. Alternatively, chronic exposure to shifts of the LD cycle could result in adaptive changes in the SCN that facilitate resynchronization following multiple shifts. These possible outcomes were tested in the present study by assessing the responses in circadian behavior and in the protein product of clock gene PER1 expression in two SCN sub-regions following four weekly phase advances. We also examined the expression of arginine vasopressin (AVP) and prokineticin2 (Pk2), two putative output signals of the SCN (Jin et al., 1999; Cheng et al., 2002). Finally, being motivated by a previous finding that in aged animals, chronic phase advances cause higher mortality rate than do chronic phase delays (Davidson et al., 2006), we further explored the responses of the SCN in rats that experienced four and eight weekly shifts of either advances or delays, to investigate the duration and direction dependent changes in the SCN. Taken together, the results suggest that rhythm perturbation could compromise the time-keeping function of the SCN and attenuate its output control, which are likely underpinnings of the internal desynchrony of the circadian system and the associated health issues.

EXPERIMENTAL PROCEDURES

Animals and housing

Male Wistar rats (28 days old) were purchased from Charles River Laboratory. The animals were housed in a 12:12 h light:dark (LD) cycle with food and water available *ad libitum*. During the day, luminescence was provided with a fluorescent white light (~300 lux at cage level). During the dark phase, a dim red light (<1 lux) was on for animal care and husbandry. After 2–3 weeks of initial entrainment period, the experimental groups went through weekly phase shifts, in which their LD cycle was shifted for 6 h every week. The control animals remained in the same LD cycle without any phase shifts. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University.

Experimental groups

Behavioral study. Animals (n=9) were singly housed in plexiglass cages (34×28×17 cm^3) equipped with running wheels (26 cm diameter, 8 cm width). Wheel revolutions were recorded in 5-min bins using VitalView (Minimitter, Inc., OR, USA). After 3 weeks of entraining to the lighting cycle and habituating to the apparatus, rats were subjected to weekly phase advances for 4 weeks, followed by 2 weeks of constant darkness (DD). The results were analyzed by ClockLab (Actimetrics, Inc., IL, USA).

Effects of four weekly phase advances on SCN function. A total of 114 rats were used in this study and half of them were exposed to weekly phase advance. At the end of the 4th weekly phase advances, the rats were perfused (see below) at Zeitgeber times (ZT) 2, 8, 11, 14, 17 and 20 (light onset time is defined as ZT0; n=6/time point) throughout a daily cycle (24 h) on day 7 of the 4th shift. Age matched control rats that did not experience any phase shifts were sacrificed at the same ZTs (n=6/time point). The brains were processed for examinination of the expression of PER1 and AVP using immunocytochemistry (ICC). Additional experimental animals and controls were perfused at either ZT4 or ZT8 (n=5–6/group/time point) to determine whether expression of *Pk2* mRNA changes in response to perturbation of the LD cycle.

Effects of the direction and the duration of the phase shifts. Rats (n=5/group) were exposed to four or eight weekly phase delays or eight weekly phase advances and then sacrificed at ZT14, the time of peak expression of PER1. Brain tissues were processed together with those from the four weekly phase advance groups and their control groups.

Transcardial perfusion

Rats were overdosed with sodium pentobarbital (200 mg/kg) and perfused intracardially with 100 ml saline followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were post-fixed for 12–18 h, and cryoprotected in 20% sucrose overnight. Alternate sets of coronal sections (40 μm) were cut through the entire SCN using a cryostat (Leica, IL, USA).

Immunocytochemistry (ICC)

Free-floating sections were incubated with mPER1 antibody (1: 5000, gift of Dr. D. R. Weaver, University of Massachusetts, MA, USA currently available as Millipore AB2201) or AVP antibody (1:10,000, Peninsula labs, CA, USA) and processed with avidin-biotin-peroxidase technique using 3,3′-diaminobenzidine (DAB) as the chromogen (Hastings et al., 1999; Yan and Silver, 2004). To deal with potential variability in independent ICC runs, animals to be compared directly were processed together with unique cut to mark each brain. After the ICC reaction, sections were mounted on slides, dehydrated with alcohol rinses, cleared with xylene and coverslipped with Permount (Fisher Scientific, NJ, USA).

In situ hybridization

The *Pk2* cDNA fragment containing plasmid was generously provided by Dr. Q. Y. Zhou, UC, Irvine (Cheng et al., 2002). *In situ* hybridization was performed as described previously (Yan et al., 1999; Yan and Silver, 2002). Briefly, sections were processed with proteinase K at 37 °C and 0.25% acetic anhydride at room temperature for 10 min. The sections were then incubated in hybridization buffer containing the Dig-labeled *Pk2* cRNA probes (0.1 μg/1 ml) overnight at 60 °C in a water bath shaker. After a high-stringency post-hybridization wash, sections were treated with RNase A, and then were further processed for immunodetection with a nucleic acid detection kit (Boehringer Mannheim, Indianapolis, IN, USA). Sections were incubated in a blocking reagent diluted 1: 100 in buffer 1 for 1 h at room temperature, then incubated at 4 °C in an alkaline phosphatase-conjugated digoxigenin antibody diluted 1:2000 in buffer 1 for 3 days. On the following day, sections were then incubated in a solution containing nitroblue tetrazolium salt (0.34 mg/ml; Roche, IN, USA) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (0.18 mg/ml; Roche) for 14 h. The colorimetric reaction was halted by immersing the sections in TE buffer (10 mm Tris–HCl containing 1 mm EDTA, pH 8.0). Sections were mounted and coverslipped as described above.

Data analysis

Behavioral data were quantified by calculating the percentage of total activity that occurred during night phase, at 2 h and 4 h intervals. The baseline was obtained by averaging the data from 7 days prior to the first shift. One-way ANOVA, followed by post-hoc Tukey HSD (Kramer) tests, was performed to assess the day-by-day changes within each week, and the temporal patterns of activity at 2 h and 4 h bins. ANOVA was also performed on the precentage data following Arcsine transformation, which revealed the same conclusion. Correlation coefficients between the baseline and the 1st or 4th shift were calculated using Pearson's r.

These correlation coefficients were then compared using Fisher's r-to-z transformation to test the significance of the differences. The activity onset on the first day in DD was calculated using the regression-based projection using the first 7 days activity in DD. For the brain data, images of serial sections through the SCN were captured using a CCD video camera (CX9000, MBF bioscience, VT, USA) attached to a light microscope (Zeiss, Gottingen, Germany). The number of mPER1-immunoreactive (ir) cells were counted bilaterally in three mid-SCN sections using the NIH Image J program, and the average was used to represent the value for each animal. The number of *Pk2* mRNA expressing cells were also counted with the same method. Using a template based on the distribution of *Avp* mRNA (Yan and Okamura, 2002), the number of PER1-ir nuclei were further analyzed in the DM and VL sub-regions separately. The number of AVP-containing cells were counted manually by two observers who were blind to the experimental conditions and the average was used to present the value for each animal. The density of AVP fibers was quantified using NIH Image J. A threshold was set arbitrarily to distinguish the fiber staining from the background, the number of pixels above the threshold in the area of interest was measured. One-way or two-way ANOVA followed by post-hoc Tukey HSD test or *t*-test alone was used to assess the effect of phase shifting on the expression of each parameter.

RESULTS

Behavioral responses following chronic phase shifts

Wheel-running activity revealed a gradual adaptation to the weekly phase shifts (Fig. 1). Actograms from two individuals are shown in Fig. 1A. Quantitative analysis confirmed that during the initial LD cycle, all rats were entrained with ~90% of the wheel-running activity occurring in the dark phase (Fig. 1B). During the first 3 days of the

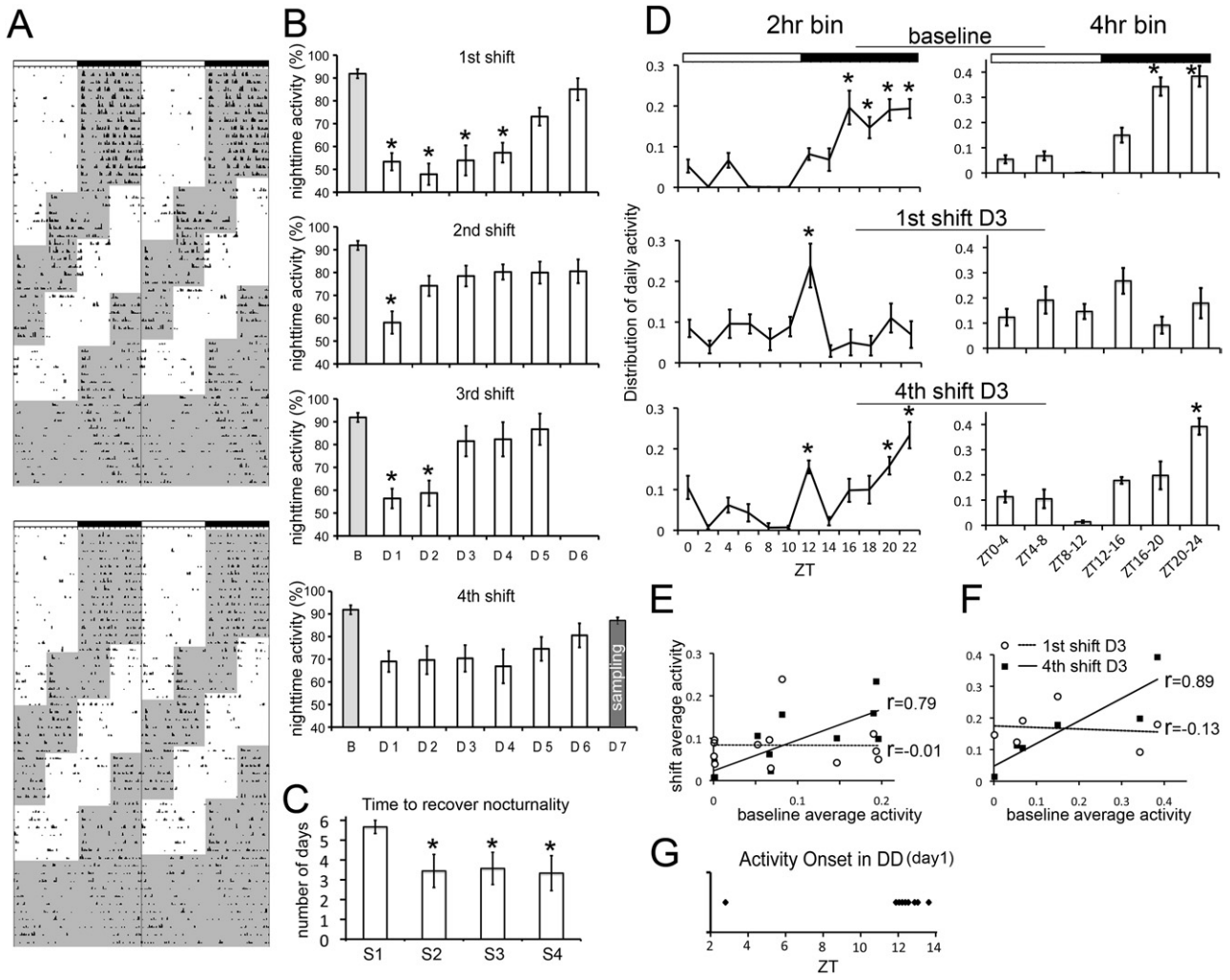

Fig. 1. Behavioral response of rats to weekly 6 h phase advances. (A) Actograms from two representative animals. The wheel-running activity was double-plotted for easy visualization. The gray shadow represents the dark phase (night). (B) Histogram shows the day-by-day nighttime activity ratio following each shift. The light gray bars on the left of each histogram show the baseline prior to the shift, the dark gray bar in the bottom panel shows the data on day 7 when the other groups of the animals were sacrificed. (The data for D6 on the 3rd shift was missing due to technical error). (C) Duration for the percentage of nighttime activity to rebound above 75% of baseline level in each animal following each shift. (D) Distribution of daily activity at 2 h bin (left panels) and 4 h bin (right panel) during baseline recording (top panels), 1st shift (mid panels) and 4th shift (bottom panels). (E, F) Correlations of the group average activity between baseline and that on the 3rd day of either 1st or 4th shift. Each data point in the scattered plot represents the group average activity at 2 h (E) and 4 h (F) intervals. (G) Activity onset time on the first day upon released to DD. The results are shown as mean±SEM, $n=9$. * Post-hoc Tukey test, $P<0.05$.

first phase advance (Fig. 1 A,B, top panel), positive masking-like activity was observed at dark onset, but no clear patterns of entrainment were observed in the animals. The predicted "complete" entrainment is 90% activity at night as seen in baseline condition. Toward the end of the week, the nocturnal activity pattern was reestablished and the nighttime activity reached above 70% on day 5 and was back to 85% on day 6. On day 7, the next shift began and the rats had to readjust to the new LD cycle. During the following shifts, the duration for the rats to reestablish their nocturnal activity pattern was faster compared to the initial one. At the 2nd and 3rd shift (Fig. 1B, two middle panels), nighttime activity reached 70% within 2 or 3 days. While for the 4th shift (Fig. 1B, bottom panel), the average percentage of nighttime activity over total activity was at 70% on the first day. And on day 7 of the 4th shift, when the animals were sacrificed for evaluating the clock protein expression in the following experiments, about 85% of the activity occurred during nighttime. A two-way ANOVA (day× week) revealed a significant interaction ($F_{14,196}=1.98$, $P<0.05$). One-way ANOVA for each week of phase shifts revealed a significant effect of the number of days in the 1st, 2nd and 3rd shift (1st shift, $F_{5,53}=8.93$; 2nd shift, $F_{5,53}=3.63$; 3rd shift, $F_{4,34}=4.09$, $P<0.05$) but not in the 4th shift ($F_{5,53}=0.72$, $P>0.05$).

To determine how long it took for the rats to reestablish their nocturnal activity pattern, the number of days for the percentage of nighttime activity to rebound above 75% of the baseline (pre-shift) level in each animal following each shift were calculated (Fig. 1C). On average, it took more than 5 days at 1st shift and about 3 days for the following shifts (one-way ANOVA: $F_{3,32}=2.99$, $P<0.05$). Post-hoc comparision revealed significant differences between the 1st and the other shifts (Tukey HSD test, $P<0.05$). The activity patterns on day 3 during the 1st and the 4th shift were compared with baseline in 2 h and 4 h intervals (Fig. 1D). A clear nocturnal pattern was observed for the baseline and for day 3 of the 4th shift, but not for day 3 of the 1st shift. Using data grouped at 2 h intervals, the activity was high in the 2nd half of the dark phase during baseline (Fig. 1D, top left panel, one-way ANOVA: $F_{11,107}=14.4$, $P<0.05$). During the 1st shift, on day 3, there was a masking-like single activity peak right after dark onset (Fig. 1D, mid left panel, one-way ANOVA: $F_{11,107}=3.41$, $P<0.05$). While during the 4th shift, the activity was high at the beginning and the end of the dark phase (Fig. 1D, bottom left panel, one-way ANOVA: $F_{11,107}=10.1$, $P<0.05$). Further, when the activity data were grouped at 4 h intervals (Fig. 1D, right panels), the one-way ANOVA revealed a significant time of the day effect for the baseline ($F_{5,53}=34.6$, $P<0.05$) and for the 4th shift ($F_{5,52}=10.29$, $P<0.05$), but not for the 1st shift group ($F_{5,53}=1.86$, $P>0.05$).

To compare directly the activity patterns during the shift with those from the baseline, correlation coefficients were calculated using data reported in Fig. 1D. Two correlation coefficients were calculated, by correlating baseline activity with 1st shift and 4th shift activity, respectively using data pooled at 2 h bin (Fig. 1E) and 4 h bin (Fig. 1F). These two correlation coefficients were found to be significantly different for the data pooled at 2 h bin (Fig. 1E, $P=0.02$) and marginally significant for that at 4 h bin (Fig. 1F, $P=0.05$).

After the 4th shift, the rats were switched into constant darkness to evaluate whether their activity rhythms were truly entrained to the LD cycle or were just masked by the LD cycle. All of the rats demonstrated free-running rhythms (Fig. 1A). The period of the free-running rhythm was slightly longer than 24 h, which is typical for this strain (Honma et al., 1985; Nagano et al., 2009). Moreover, in eight out of nine rats the free-running rhythms had onsets close to the projected ZT12 (Fig. 1G). The activity onset times on the first day of DD are shown in Fig. 1G. The results suggest that on an average, the activity rhythms were generally entrained at the end of the shifts and that interindividual differences were minimal.

PER1 rhythms in the SCN following weekly phase advances

Rhythmic PER1 expression was observed in the SCN of animals from both control and shift groups (Fig. 2). As shown by the photomicrographs, in the control group (Fig. 2A, upper panels), the peak expression of PER1 protein was observed at ZT11 and 14, and the nadir was at ZT2 and 20, consistent with previous findings (Field et al., 2000; Nuesslein-Hildesheim et al., 2000; Sumova et al., 2002). After four weekly shifts (Fig. 2A, lower panels), the circadian temporal profile of PER1 was similar to that of the control group.

The number of PER1-ir nuclei in the entire SCN (Fig. 2B) was analyzed by two-way ANOVA, which revealed a significant interaction between ZT and shift ($F_{5,71}=2.52$, $P<0.05$). Post-hoc comparisons revealed a significant difference between the control and the shift group at ZT14 ($P<0.05$). The number of PER1-ir nuclei was further analyzed in the SCN sub-regions. In the dorsomedial (DM) SCN, two-way ANOVA revealed a significant interaction between time of day and phase shift ($F_{5,71}=3.96$, $P<0.05$). Post-hoc comparisons revealed significant differences between control and experimental groups at ZT11 and 14 ($P<0.05$) and a marginally significant difference at ZT17 ($P=0.052$). In the ventrolateral (VL) SCN, there was no significant difference between the two groups (Figs. 2D, two-way ANOVA, effect of ZT: $F_{5,71}=69.25$, $P<0.05$; effect of shift: $F_{1,71}=0.65$, $P>0.05$; interaction: $F_{5,71}=1.09$, $P>0.05$).

Output signals from the SCN following weekly phase advances

The expression levels of the SCN output signals AVP and *Pk2* were examined in the 4th advance group and the controls. AVP level (Fig. 3) was examined in the SCN and in the ventral sub-paraventricular zone (vSPVZ), the major SCN target (Stephan et al., 1981; Watts et al., 1987) at ZT2 and 14, the peak and trough time of the peptide (Tominaga et al., 1992). At ZT2, the number of AVP-containing cells was not significantly different between the control and shift group (*t*-test, $P>0.05$; Fig. 3B). However,

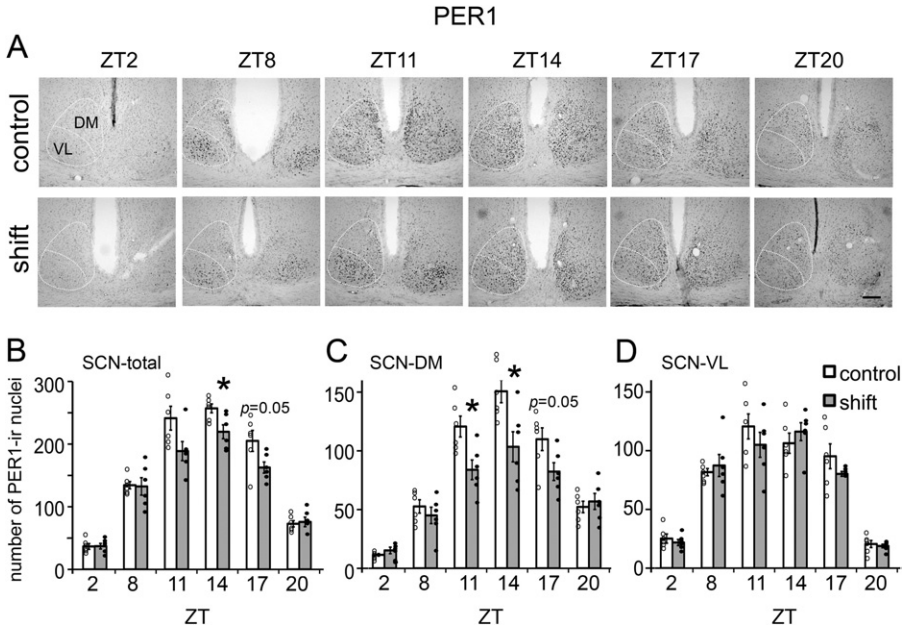

Fig. 2. PER1 expression levels in the SCN of control and shifted animals. (A) Representative photomicrograph showing PER1 staining in the SCN. White dashed lines delineate the DM and VL SCN sub-regions. Scale bar, 100 μm. Histograms show quantitative analysis of PER1-ir nuclei in the entire SCN (B), DM (C) and VL (D) sub-regions in the two groups. The results are shown as mean±SEM, n=6. The open and closed circles plot the data from individual animals in the control and shift groups, respectively. * Post-hoc Tukey test, $P<0.05$.

the AVP contents in the VL SCN and in the vSPVZ were significantly lower in the shift group (t-test, $P<0.05$; Fig. 3C, D). At ZT14, the number of AVP-containing cells were lower in the shift group (t-test, $P<0.05$; Fig. 3B‘), while the peptide contents in the VL SCN and vSPVZ were not significantly different between the two groups (t-test, $P>0.05$; Figs. 3C‘, D‘).

Pk2 expression was also examined at ZT4, 8 and 14 (Fig. 4), the peak, median and trough time for its mRNA expression (Cheng et al., 2002). The expression of *Pk2* was lower in the shift group compared to the control (Fig. 4A). Two-way ANOVA revealed a significant interaction between time and shift ($F_{2,23}$=12.5, $P<0.05$). Post-hoc comparisons revealed significant difference in the number of *Pk2*-expressing cells between the control and shift group at ZT4 and 8 (Tukey HSD test, $P<0.05$; Fig. 4B).

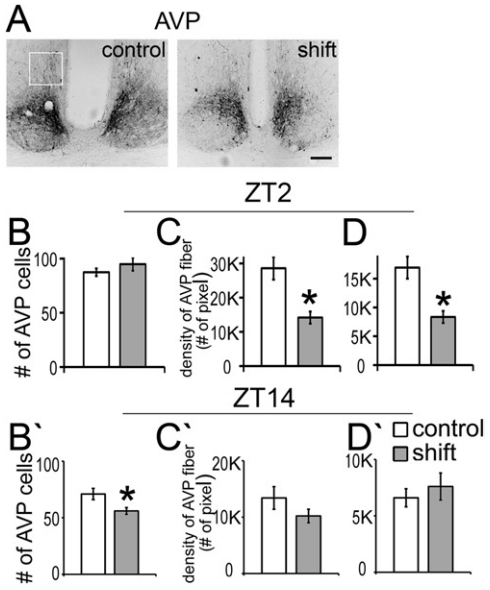

Fig. 3. Expression of SCN output signal AVP in the control and shift groups. (A) Representative photomicrographs of AVP staining in the SCN and in vSPVZ at ZT2. Outlines indicate the area for measuring AVP fiber density. (B, B′) Number of AVP-ir cells in the SCN at ZT2 and ZT14. (C, C′) Density of AVP fiber staining at ZT2 and ZT14 in VL SCN where no AVP cell bodies are present. (D, D′) Density of AVP fiber in vSPVZ at ZT2 and ZT14. The results are shown as mean±SEM, n=5–6. * t-test, $P<0.05$.

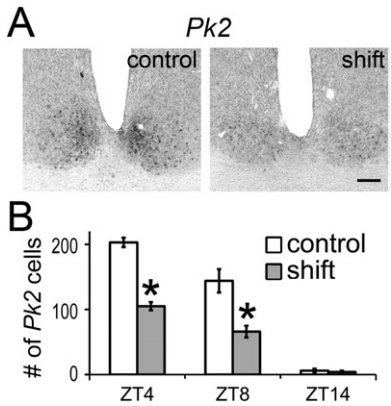

Fig. 4. Expression of SCN output signal *Pk2* expression in the control and shift groups. (A) Representative photomicrographs of *Pk2* staining in the SCN. (B) Number of *Pk2* mRNA containing cells in the SCN of control and shift group. The results are shown as mean±SEM, n=5–6. * Post-hoc Tukey test, $P<0.05$.

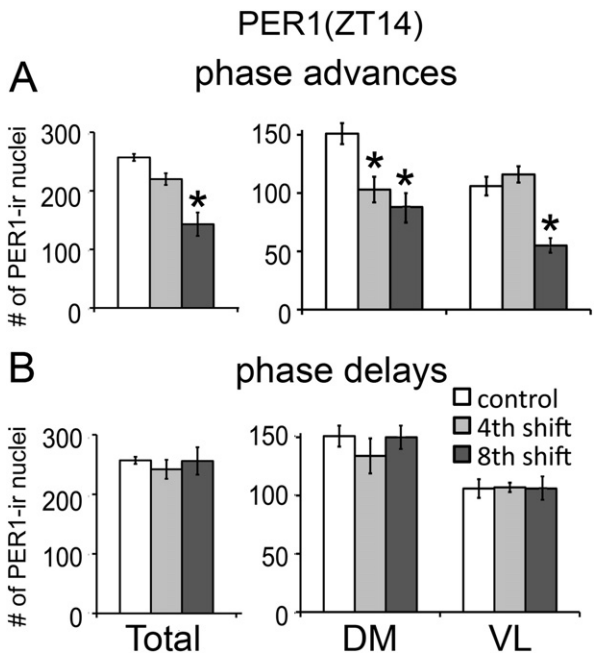

Fig. 5. PER1 expression in the SCN following multiple shifts. (A) 8 weekly phase advances cause further suppression in PER1 expression in the entire SCN (left panel) and each sub-regions (right panel). The data for the control and 4th advance group were re-plotted from that in Fig. 2. (B) Weekly phase delays have no apparent effect on PER1 expression in the SCN. The results are shown as mean±SEM, n=5–6. * Post-hoc Tukey test, $P<0.05$.

The responses of the SCN depend on the duration and direction of the phase shifts

We next tested whether longer shifting could further affect the PER1 expression in the SCN and whether the direction of the shifts could make any difference in the responses (Fig. 5). After 8 weeks of consecutive weekly phase advances, the level of PER1 expression at ZT14 was significantly reduced relative to the 4th week (Fig. 5A, left panel, one-way ANOVA: $F_{2,15}$=21.23, $P<0.05$). While the reduction in PER1 was mainly in the DM SCN at the 4th advance, at the 8th advance, PER1 levels were lower in both the DM and VL SCN of phase shifted animals compared to controls (Fig. 5A, right panel). Two-way ANOVA revealed significant interaction between shift and sub-region ($F_{2,31}$=4.78, $P<0.05$). Tests of simple main effect revealed a significant effect of shifts in both VL and DM region (VL: $F_{2,15}$=15.06, $P<0.05$; DM: $F_{2,15}$=7.10, $P<0.05$). In contrast, there were no effects of either 4 or 8 weeks of delays on PER1 expression and the delayed groups did not differ from each other (Fig. 5B). This was the case for the entire SCN (one-way ANOVA, $F_{2,15}$=0.39, $P>0.05$) as well as its sub-regions (two-way ANOVA, effect of shift: $F_{2,29}$=0.40, $P>0.05$; effect of region: $F_{1,29}$=22.71, $P<0.05$; interaction: $F_{2,29}$=0.49, $P>0.05$).

DISCUSSION

In the present study, we examined the effect of chronic jet-lag on overt rhythms and on the clock functions within the SCN. The results in behavior and in the SCN collectively suggest that the circadian system is very plastic in its responses to perturbations of the photic environment. The results also suggest that plastic responses in the SCN facilitate the resynchronization of the SCN sub-regions following multiple shifts. However, the plasticity comes at the cost of an apparent attenuation of the amplitude of circadian oscillation in the SCN and its output control. The responses of the SCN are dependent upon the duration and the direction of the phase shifts. While the repeated phase delays appear to be less disturbing, the repeated phase advances attenuate the amplitude of the daily rhythm of PER1 and compromise the output control of the SCN.

After each weekly phase advance, the wheel-running rhythms gradually get readjusted to the new phase (Fig. 1). Interestingly, the duration of the readjustment became shorter following the initial shift, suggesting adaptive responses occurred in the system. Once the rats were released into DD, their closely clustered onset times of the free-running rhythms confirmed that the majority (~90%) of the rats were re-entrained to the preceding LD cycle, which therefore validates the approach of the subsequent experiments investigating the functional responses of the SCN.

Using PER1 as a marker, the results revealed that, in agreement with the behavioral data, the overall circadian oscillation of the SCN was retained by the end of the four consecutive weekly phase advances, and that the two sub-regions displayed their original phase relationship (Fig. 2). The animals used for studying the responses in the SCN had no access to wheels, whereas the behavioral data were collected using running wheels, which may affect the effects of the shift. The SCN neurons are heterogeneous in their topography, neuropeptide content, neural afferent/efferent connections and clock gene expression, and are organized into functionally distinct sub-regions (Ibata et al., 1999; Lee et al., 2003; Antle and Silver, 2005; Yan et al., 2007). The circadian oscillations within the DM and VL sub-regions of the rat SCN are generally synchronized with each other (Yan and Okamura, 2002; Bendova et al., 2004). However, the dissociation between the two sub-regions can be induced under experimental lighting conditions (Nagano et al., 2003; de la Iglesia et al., 2004). Following a single 6 h advance, the VL and DM sub-regions of the SCN re-entrain to the new LD cycle at different rates, in 5 days or 13 days, respectively (Nagano et al., 2003; de la Iglesia et al., 2004). Therefore, under our experimental paradigm of shifting every 7 days, theoretically these two SCN sub-regions could further dissociate from each other, however that was not the case (Fig. 2). Thus, the synchrony between the two regions, which is preserved after multiple shifts is likely due to plastic changes of the SCN network. Although the experimental design of sampling every 3 h limited our ability to detect smaller phase differences, the results clearly support the adaptive plasticity hypothesis, as the two SCN sub-regions are re-synchronized by day 7 following the 4th advance. The mechanisms underpinning the faster readjustment of

behavioral rhythms following multiple shifts warrant further investigation. It is possible that during the repetitive weekly phase advances, adaptive responses occurred in the SCN network. This could involve structural and/or functional changes that alter the connectivity of the neural network, resulting in strengthening/weakening of the intercellular coupling. These changes would ultimately facilitate the synchronization between the sub-regions and between the whole SCN and the environmental LD cycle.

Although the synchrony between the VL and DM regions was re-established by day 7, there was a reduction in the amplitude of the rhythm in PER1 expression in the DM SCN. Given the behavioral data showing that the circadian phases of eight out of nine rats were tightly clustered when released in DD (Fig. 1D), the diminished amplitude of the SCN rhythms does not simply reflect greater individual variability in the phase of the individuals' SCN. Thus, it is likely that this attenuated expression of PER1 is caused by a less coherent group of oscillators in the DM SCN. It is possible that although the DM sub-region as a whole is in phase with the VL and with the LD cycle, some of the individual oscillators in DM region may still undergo further shifting and may have not been settled by day 7 to the phase of the LD cycle as it is the case in the steady-state. This could be a result of weaker or incomplete coupling between the DM and VL SCN. It has been shown that reduction in amplitude can facilitate the phase shifting effect of external stimuli (Vitaterna et al., 2006), therefore, the amplitude reduction seen here could be an adaptive response to promote synchrony between the DM and the VL SCN. Future studies should investigate how long it takes for the SCN to fully recover after prolonged rhythm perturbation, and whether there are any long-term consequences of such perturbations for the circadian system or for physiological functions controlled by the circadian system.

The SCN conveys temporal information to the rest of the brain and body through output signals (Kalsbeek and Buijs, 2002). Two putative output signals AVP and *Pk2* were examined to assess the effect of chronic phase shifts on SCN output control (Jin et al., 1999; Cheng et al., 2002). AVP plays critical roles in the promotion of several neuroendocrine rhythms (Tousson and Meissl, 2004; Gerhold et al., 2005; Christian and Moenter, 2008). The transcription of *Avp* mRNA is under direct circadian control and the AVP peptide is secreted from the SCN in a rhythmic manner (Schwartz and Reppert, 1985; Jin et al., 1999). The release of AVP is correlated with the peptide content of the SCN, as both peak in early daytime, around ZT2 (Schwartz and Reppert, 1985; Tominaga et al., 1992). At ZT2, the number of AVP-containing neurons in the SCN were not significantly affected. However, the level of AVP contained within the fibers was lower in the SCN of the shifted animals. The decreased AVP was also observed in the vSPVZ, one of the major targets for the SCN output (Stephan et al., 1981; Watts et al., 1987). At ZT14, when AVP contents and its release are at baseline level, the number of AVP neurons were lower in the shift group. *Pk2* is another putative output signal from the SCN, which has been implicated in the regulation of circadian rhythms in various behavioral, neuroendocrine and autonomic functions (Zhou and Cheng, 2005). There was a substantial reduction in the level of *Pk2* mRNA in the shifted group at the peak and median time, but no difference was seen at the baseline level. Taken together, the AVP and *Pk2* results suggest the presence of an attenuated output control from the SCN of the shifted group. This could have a negative impact on the otherwise-stable phase relationship among extra-SCN oscillators, thus affecting the overall synchrony of the circadian system.

The effects of rhythm perturbation on the SCN depend on the duration and the direction of the shifts. When the duration of the weekly advances was extended from 4 to 8 weeks, PER1 expression was further depressed in both VL and DM sub-regions, suggesting continued deterioration in SCN function as animals endure multiple shifts. It would be informative to know if or how behavioral rhythms change over this longer period. We were unable to collect behavioral data for a longer period because the rapid growth of the animals impeded the use of running wheels to monitor activity rhythms. Therefore we do not know if the rhythms were still capable of entraining to the shifted LD condition or if the rhythms persisted through the end of the 8th phase advance. However, the pattern and number of PER1-ir cells in the SCN were relatively consistent among individuals within the group, suggesting that the rats were likely at the same circadian phase and were entrained to the LD condition. But we cannot completely rule out the possibility that the animals may have become arrhythmic with constant low expression of PER1. In either case, the results indicate that there are limitations in the adaptive responses of the SCN neural network. It should be noted that the rats used in the study were young (~50 days old) when the first phase shift was introduced, which could have contributed to the seemingly effective plastic responses during the first four phase advances (Schulz et al., 2009). It is possible that mid-aged or aged animals would show less plastic or adaptive responses both behaviorally and neurologically under the same treatment.

In contrast to the phase advances, the weekly phase delays appear to be less disruptive for the SCN. The neural mechanisms underlying the different responses in repetitive phase delays and advances warrant further investigation. After a single 6 h shift in the LD schedule, Reddy et al. (2002) showed that the stable re-entrainment of mouse behavioral rhythms takes at least 7 days for advances and only 3 days for delays. Furthermore, rhythms in the clock genes *Per* and *Cry* in the mouse SCN were dissociated during a phase advance, but shifted in a coordinated fashion following a phase delay. With a detailed analysis of clock gene expression in VL and DM regions in the rat SCN, Nagano et al. (2003) demonstrated that the resynchronization between the two sub-regions is archived in 10–13 days following a 6 h advance, but in only 7 days following a 10 h delay of the LD cycle. All the results suggest that it is easier for the SCN to adjust to phase delays than to advances. It should be noted that the Wistar rats have free-running periods longer than 24 h. Therefore

the effective daily shift required in the phase advance paradigm is larger than in a phase delay paradigm, which could account for the observed differences between the two paradigms. Alternatively, phase delays and advances could be associated with changes in other clock genes/proteins, as suggested by previous studies (Reddy et al., 2002; Yan and Silver, 2002, 2004).

In the United States, millions of people work on a shifting schedule or suffer from sleep disorders every year (Roldan and Ang, 2006). There are widespread concerns about the long-term health impact derived from these conditions (Haus and Smolensky, 2006). A recent study using a mouse model under chronic jet-lag paradigm has shown that circadian disruption, independent from sleep loss or stress, can lead to dysregulation of the immune system (Castanon-Cervantes et al., 2010). Our results on the SCN responses following repeated phase shifts provide evidence linking the SCN function and the ultimate health consequence following circadian rhythm perturbation, and support the hypothesis that the health issues related to shift work and sleep disorders are derived from deficits in SCN function.

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