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Immunosuppressant calcineurin inhibitors phase shift circadian rhythms and inhibit circadian responses to light

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ABSTRACT

PP2B is a Ca^{2+}/calmodulin-dependent protein phosphatase that is ubiquitously expressed in mammals. Among other actions, it is an effector mechanism in NMDA-mediated glutamate neurotransmission as well as a regulator of GSK3β and MAPK signaling cascades. Because all of these mechanisms have demonstrable roles in the control of circadian rhythyms, we hypothesized that PP2B would be a key regulator of rhythm generation and entrainment, and that through inhibition of its phosphatase activity, the circadian system would be affected by immunosuppressant drug therapy. We report here that immunosuppressant drugs (cyclosporin A, FK506) (1) block the circadian responses to light that underlie photic entrainment; (2) produce circadian phase shifts with a characteristic nonphotic profile; and (3) disrupt circadian rhythm expression when applied chronically. These results indicate a role for PP2B in circadian rhythm generation and entrainment. In addition, because rhythm disturbance has been implicated in impairment of both physical and mental health, we suggest that the use of immunosuppressants would be safer and more efficacious if their impacts on circadian rhythmicity were taken into account.

1. Introduction

Calcineurin (PP2B) is a Ca^{2+}/calmodulin-dependent protein phosphatase that is ubiquitously expressed in the mammalian central nervous system (Klee et al., 1979; Rusnak and Mertz, 2000). PP2B inhibitors cyclosporin A (CsA) and tacrolimus (FK506), are the most widespread immunosuppressants used in medicine. They bind to cytosolic immunophilins forming complexes that inhibit PP2B phosphatase activity, leading to significant decreases in cytokine synthesis (Liu et al., 1991; Schreiber and Crabtree, 1992). The therapeutic use of these drugs is often associated with adverse side effects that decrease the patients' quality of life. These include disorganization of sleep and wake patterns (Neuhaus et al., 1994; Kemper et al., 2003), and effects on circadian physiology, such as the decrease in the normal nocturnal dip in blood pressure, which is linked to an altered cardiac function (Lipkin et al., 1993; Taler et al., 1995; van den Dorpel et al., 1996; Galiatsou et al., 2000). The mechanisms underlying such disturbances are not clear. In the case of circadian variations in blood pressure, the absence of day/night rhythms associated with CsA treatment has been linked to reduced corticosteroid rhythmicity, decreased endothelial nitric oxide synthase activity in the peripheral circulation, and impaired renal function (van de Borne et al., 1993; van den Dorpel et al., 1996; Kooman et al., 2001; Curtis, 2002). The mechanisms that trigger sleep disturbances are not known.

The reduction in day/night variation in physiology and behavior may be due to a direct effect of immunosuppressants on the central circadian clock. Circadian rhythms are produced by a hierarchy of circadian oscillators and clocks in central and peripheral tissues. At the top of this hierarchy are circadian pacemaker cells located in the hypothalamic suprachiasmatic nucleus (SCN) (Welsh et al., 1995; Klisch et al., 2006).

The chemical target of immunosuppressants, cyclophilins and immunophilins, participate in the regulation of intracellular Ca^{2+} channels as well as the activity of PP2B. The SCN circadian clock is sensitive to immunosuppressants that interfere with intracellular Ca^{2+} storage and release (FK506, rapamycin; Ding et al., 1998). The molecular basis of the clock involves the transcriptional and translational feedback loop activity of several genes (including *per1*, *per2*, *cry1*, *cry2*, *clock* and *bmal1*), which determines their cyclic activity with a period close to 24 h (see Dunlap, 1999; Reppert and Weaver, 2002). Clock genes are regulated by post-translational modifications of proteins in each, including phosphorylation by protein kinases CKIε and CKIδ (Vielhaber et al., 2000; Eide and Virshup, 2001), and GSK3β (Iitaka et al., 2005; Yin et al., 2006). Indeed, PP2B is a key regulator of casein kinase-Iε (CKIε) and GSK3β (Cegielska et al., 1998; Lowrey et al., 2000; Liu et al., 2002; Fiedler and Wollert, 2004).

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In addition, photic entrainment of circadian rhythms in the SCN requires glutamate signaling from the retino-hypothalamic tract (RHT) and the subsequent activation of NMDA and non-NMDA receptors. This leads to an increase of cytosolic Ca^{2+} levels, which in turn activates Ca^{2+}/calmodulin-dependent processes, including PP2B activity. Thus, PP2B is in a position to regulate both the photic responses of the circadian system as well as the rhythm generation mechanism in the SCN. In this paper we test the ability of PP2B inhibitors to alter circadian rhythms in the Syrian hamster, a well-known animal model for chronobiology.

2. Materials and methods

2.1. Animals and experimental procedures

Syrian hamsters (*Mesocricetus auratus*) were raised in our colonies and were housed under a 24 h light–dark cycle with 14 h of light per day (LD14:10). Food and water were available *ad libitum*, and a running wheel was freely available in all experimental conditions. Male adult (4–6 months) animals were used in all experiments. In LD conditions, clocktime refers to *Zeitgeber* time (ZT) with ZT 12 defined as the time of lights off. In constant dark conditions (DD) *circadian* time (CT), was used as a reference with CT 12 defined as the onset of locomotor activity. The phase of circadian locomotor activity in DD was determined using eye-fit lines drawn through consecutive onsets of activity as estimated by three observers blind to experimental manipulations. Circadian phase shifts were determined using lines fit to the activity onsets on the seven days prior to the treatment and the 4th to the 10th days after treatment. The phase shift is the difference between the timing of the expected and observed onsets immediately following the treatment as indicated by these lines.

For time-scheduled neurochemical determinations in DD, animals were entrained to the LD14:10 schedule, then were switched to DD for 48 h and sacrificed at 4-h intervals. A dim red light source (<1 lx) that does not cause phase shifts nor disturb freerunning circadian rhythms was used as an aid in DD manipulations.

All procedures were performed according to NIH standards for animal care.

2.2. Locomotor activity monitoring

Hamsters were housed individually in cages equipped with a 17 cm diameter running wheel and their circadian rhythms of wheel-running activity were recorded continuously using Dataquest III (Minimitter Co. Inc., Bend, Oregon).

2.3. Surgical procedures

For cannulation, hamsters were deeply anesthetized with 75 mg/kg ketamine and 10 mg/kg xylazine. Stainless steel guide cannulae 22 ga. (Plastics One, Roanoke, VA) for intracerebroventricular (i.c.v.) injection, were implanted into the third ventricle (0.6 mm anterior to bregma and 8.2 mm ventral to the skull surface directly on the midline, with the tooth bar set at −2.0 mm). Dummy cannulae were inserted into the guide tubes to prevent blockage. After recovery from anesthesia, animals were housed in constant darkness and monitored for two weeks before any experimental procedure was performed. Injections were made through the guide by removing the dummy cannula and inserting an injector that extended about 1 mm beyond the tip of the guide when fully seated. Infusions of 2 μl were made at a flow rate of 1 μl/min.

In order to assess the effect of chronic treatment with PP2B inhibitors, we used Alzet 2002 osmotic pumps (Durect Corporation, Cupertino, CA), which release their contents (200 μl) at a rate of 1 μl/h over 14 days. Cannulae were placed by stereotaxic surgery aimed at the third ventricle, and upon recovery of the animals locomotor activity was recorded for at least 10 days. After this initial period, hamsters were anesthetized under dim red light with isoflurane and a local injection of xylocaine in order to place the subcutaneous osmotic pump (filled with FK-506 or saline) and a 4 cm-long silicone tubing connected to the i.c.v. cannula. Each surgery for the placement of the minipump took less than 30 min to perform, and was conducted during the animals' activity phase (after CT12) to avoid phase shifts related to the procedure.

2.4. Drugs

CsA was purchased from Sigma-Aldrich Co. (St. Louis, MO), and was diluted in DMSO. FK506 and its vehicle were a generous gift from Fujisawa Pharmaceuticals Co., Ltd., (Osaka, Japan).

2.5. Neurochemical analyses

For Western blot analysis, animals were decapitated and micro-punches containing the SCN were obtained from hypothalamic slices. Tissue was homogenized in 25 mM Tris–HCl buffer, pH 7.4, containing 0.32 M sucrose, 1 mM EGTA, 1 Mm EDTA, 50 mM NaF, 2 mM Na_3VO_4, 0.03 mM okadaic acid and a protease inhibitor cocktail for mammalian tissue (Sigma-Aldrich Co., St. Louis, MO). Protein concentration was measured by the Lowry method; 40 mg of protein were separated in 9% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, UK). Control of transference and equal loading was performed with reversible Ponceau S staining. After blocking with 10% non-fat dried milk in Tris buffer saline-Tween 20 for 1.5 h at room temperature, membranes were incubated 4 h with 1/1000 anti-PP2B antibody (Santa Cruz Biotechnology Inc., California) and 1 h with 1/4000 secondary HRP-labeled antibody (BioRad, California,). Immunochemical detections were performed by enhanced chemioluminescence (Amersham Biosciences, UK) on a X-ray film (Agfa-Gevaert S.A., Argentina).

PP2B activity in the SCN was measured in micropunches pooled from 7 animals/point, obtained as described above. Homogenization and phosphatase activity measurement were performed according to the specific colorimetric assay kit AK-816® (Biomol Int. L. P., PA). In summary, the system measures cellular calcineurin phosphatase activity by means of the release of free-phosphate from the RII phosphopeptide substrate, which is detected by a non-radioactive Malachite green assay. Each experiment was repeated three times.

2.6. Statistical analysis

Comparisons among means of independent groups were performed with either Student's *t* test or ANOVA or, when cross-over protocols were performed, paired *t* test and repeated measures ANOVA were used. For the analysis of CSA-induced phase shifts, two-way ANOVA was performed with time and treatment (CsA or vehicle) as factors. Post ANOVA comparisons between groups were performed with Tukey's test for one-way ANOVA and with Bonferroni's test in the other cases. The analysis of robustness of circadian locomotor activity rhythm was performed by the combination of visual inspection and computation of activity counts during normal “resting” or “active” hours as defined by the previous locomotor pattern of the animals. In addition, circadian period was determined by Chi-square periodogram analysis, and the Qp value for each spectrum was used for an additional calculation of the robustness of the rhythms. Data are presented as mean±SEM, and statistical significance of differences was defined when $p<0.05$.

3. Results

3.1. PP2B inhibitors block circadian responses to light

Circadian phase shifts induced by light at night are mediated by NMDA receptor activation. Because the subsequent increase in cytosolic Ca^{2+} results in the activation of PP2B (Alagarsamy et al.,

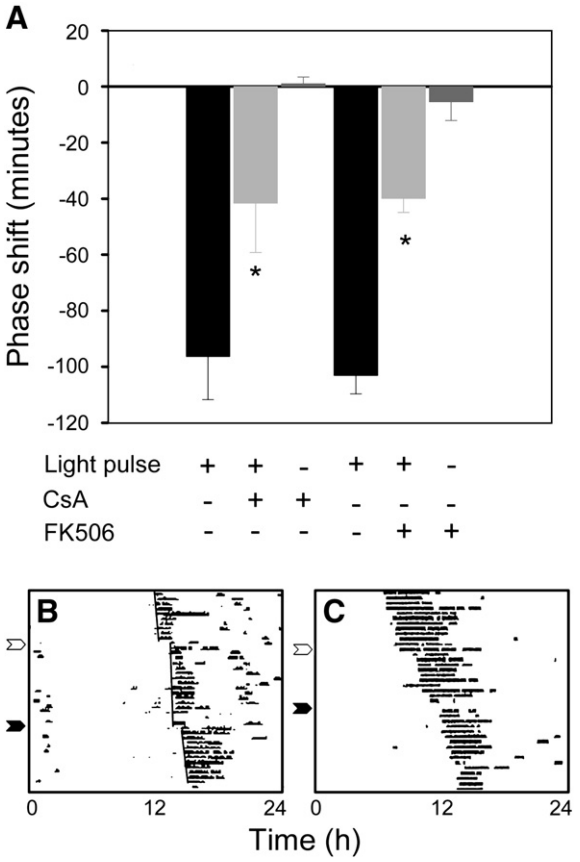

Fig. 1. Inhibition of light-induced phase shifts by CsA or FK506 in constant darkness. *Upper graph*, effect of 80 mg/kg CsA i.p. or 1 mM FK506 i.c.v. applied 30 or 15 min prior to 15 min 50 lx light pulse at CT13.5 (1.5 h after onset of activity phase), respectively. Positive values in *Y* axis correspond to phase advances and negative values to phase delays ($n=6$ per condition, repeated measures ANOVA test: $p<0.01$ for CsA, $p<0.0001$for FK506; post-ANOVA Bonferroni's test: ***$p<0.01$ vs light pulse/vehicle administration). *Lower graphs*, representative actograms of animals treated with (B) CsA or (C) FK506 (black arrows) and their respective vehicles (white arrows) prior to a 50 lx, 15 min light pulse at CT13.5. Consecutive days are plotted below each other along *Y* axis. Phase of circadian locomotor activity is shown with black line for days pre- and post-injection.

2005), we determined whether this action participates in the setting of circadian phase by light. Hamsters freerunning in DD were administered either 80 mg/kg CsA (i.p.) or 1 mM FK506 (i.c.v.) or respective vehicle controls, 30 min (CsA) or 15 min (FK506) prior to a phase-delaying light pulse at CT13.5 (15 min, 50 lx). CsA significantly reduced the effect of light by 64.1±15.8% and FK506 by 63.8±4.4% with respect to control injections at this time point. Neither drug had any effect by itself on circadian phase (Fig. 1).

3.2. Acute administration of PP2B inhibitors induces nonphotic-like phase shifts in circadian locomotor activity rhythms

We examined the potential effects of PP2B inhibition on circadian phase. The i.p. administration of 10 mg/kg of CsA at different circadian times to hamsters housed in constant darkness resulted in circadian phase shifts that exhibited a nonphotic temporal profile (i.e., phase shifts during the circadian day). Phase advances were obtained during the subjective day with the peak response (31.25±6.18 min) occurring at CT6. A higher dose (100 mg/kg) induced advances of 83.09± 7.87 min at the same time point (Fig. 2). Higher doses (200 mg/kg) appeared unhealthy for the animals.

To determine whether these changes were due to direct effects on the SCN, we administered CsA at CT6 (5 mM i.c.v.) and obtained significant phase advances of 32.74±6.30 min, compared with vehicle injection (9.67±6.39 min) ($p<0.05$, paired Student's *t* test). In addition,

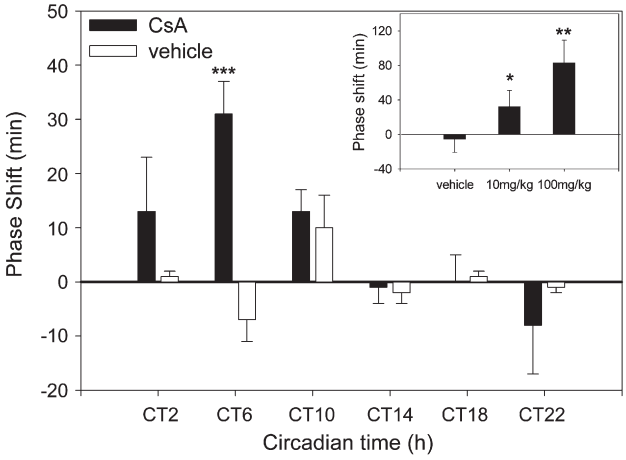

Fig. 2. Phase shifts in circadian rhythm of locomotor activity induced by CsA i.p. administration in constant darkness. *Upper graph*, phase response curve for the i.p. administration of 10 mg/kg CsA or vehicle. Animals were injected under DD at different times of their locomotor activity endogenous cycle, and the resulting phase shifts were calculated from days 4–10 post-injection. CT (circadian time) is referenced to the onset of activity phase defined as CT12. Positive values in *Y* axis correspond to phase advances and negative values to phase delays. (Two-way ANOVA: $p<0.01$ for factor *time*, $p<0.02$ for factor *treatment*, $p<0.001$ for *interaction*; ***post-ANOVA Bonferroni's test: $p<0.05$ for CsA CT6 vs CsA CT14, 18, 22 and vehicle CT6) ($n=4$–7 per treatment). *Inset graph*, dose-dependent phase shifts for i.p. administration of CsA at CT6 (ANOVA test: $p<0.0001$; post-ANOVA Tukey's test: *$p<0.01$, **$p<0.001$) ($n=6$ per dose).

1 mM FK-506 induced small, albeit significant phase advances at CT 6 (25±14 min, $p<0.05$ vs vehicle, Student's one tail *t* test).

3.3. Effects of CsA on PP2B presence and activity in the SCN

We performed western blots to characterize the diurnal and circadian profile of PP2B in homogenates of SCN. We found a specific band immunoreactive for calcineurin, but no significant changes with time of day were evident neither under a light/dark schedule nor in constant darkness (data not shown).

To confirm that suprachiasmatic PP2B is inhibited by the peripheral administration of its blockers, we measured its activity in the SCN after CsA intraperitoneal (i.p.) administration. We injected 80 mg/kg CsA at CT6 and sacrificed animals 0, 30 and 60 min later. A significant transient decrease in PP2B activity was found 30 min after treatment (Fig. 3).

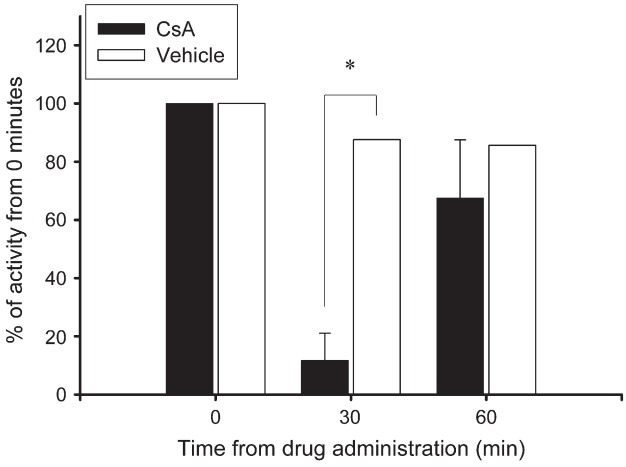

Fig. 3. Effect of 80 mg/kg CsA i.p. at CT6 on PP2B phosphatase activity in SCN homogenates 0, 30 or 60 min after drug or vehicle injection. The percentage of activity (with 100% corresponding to 0 min) is shown (*$p<0.05$, Student's test, $n=3$ per condition).

Table 1
FK-506 infusion increases locomotor activity in the subjective day

	Circadian period (min)			Subjective day activity		
	Pre-pump	During pump	Post-pump	Pre-pump	During pump	Post-pump
Vehicle	1447±14	1450±14	1435±17	6.1±5.7	6.1±2.9	4.1±3.9
FK-506	1441±10	1455±17	1428±12	7.9±0.7	29.8±8.6 *	20.5±5.2

Circadian period (analyzed by Chi-square periodograms) was not affected by chronic infusion of vehicle or FK-506. The percentage of subjective day (defined from the 10 days previous to pump implantation) locomotor activity with respect to total activity counts before, during or after the effects of a miniosmotic pump delivering vehicle or FK-506 into the SCN is shown ($n=4$). ANOVA: $F=4.09$, $p<0.025$. (*)$p<0.05$ vs. FK-506 pre-pump values.

3.4. Chronic PP2B inhibition disrupts circadian locomotor activity

Immunosuppressants are invariably applied continuously over extended periods of time in human patients. To assess the effects of chronic inhibition of SCN PP2B on the circadian clock, we performed i.c.v. constant perfusion of FK506 with an osmotic pump placed subcutaneously in the back of the animal, connected to an i.c.v. cannulae via a short silicon hose. Although no changes in circadian period were found (Table 1), during two weeks of perfusion, the animals changed their pattern of circadian locomotor activity, with an increased alpha (activity phase length) and a clear tendency towards arrhythmicity. In order to analyze these changes, we measured locomotor activity during the "resting" hours (subjective day, as defined for the 10 pre-pump days) in relation to total activity and found no changes for vehicle-treated animals and an increase in subjective day activity during CsA treatment that tended to be restored after the end of perfusion. In addition, we computed the Qp value of each Chi-square periodogram analysis as an additional index of rhythm robustness. While vehicle-treated animals exhibited no change in their Qp values before and after infusion (pre-pump 277±20; during pump 290±52; post-pump 270±27), Qp decreased significantly in FK-506-treated hamsters (pre-pump 490±70, during pump 240±46, $p<0.01$, Tukey test). This decrease in Qp was not reversed after infusion stopped (270±74, $p<0.05$ vs pre-pump values, Tukey test). No consistent changes in other circadian parameters were observed (Fig. 4, Table 1). After the experiment the animals were sacrificed to verify cannulae injection site; in addition, histological observations indicated no tissue necrosis had been developed.

4. Discussion

We have found that immunosuppressant drugs alter circadian rhythm generation and entrainment in three ways that could account for the deleterious effects often reported for human patients on immunosuppressant therapy. The effects on circadian phase reported here resemble those produced by stimuli that are classified generally as "nonphotic". Most nonphotic stimuli inhibit responses to light during the subjective night and induce circadian resetting during the subjective day (in nocturnal organisms) (Yannielli and Harrington, 2004). We found that immunosuppressants (1) block circadian responses to light at night; (2) produce phase advances of the circadian rhythm when applied during the subjective day; and (3) disrupted circadian rhythm expression when applied chronically. In addition, the amount of wheel running during the subjective day (which is typically low under normal conditions) was increased during drug infusion, thus decreasing the robustness of the rhythm during calcineurin inhibition. These results suggest that PP2B in the SCN is a target of immunosuppressant drugs and plays a role in the signal transduction pathway of the circadian clock, and in the absence of light influences the circadian cycle during the subjective day.

The findings suggest a critical role of PP2B in intracellular photic signal transduction, a pathway which has been shown to activate Ca^{2+}/calmodulin-related enzymes (such as CaMKII and nNOS) (Agostino et al., 2004) and to change the phosphorylation state of clock proteins (Reppert and Weaver, 2002). This pathway is also related to long-term potentiation in the SCN, which has been reported to be modulated by CsA (Fukunaga et al., 2002). Since we found a time-dependent effect of PP2B inhibition, it could be speculated that basal levels of PP2B activity exhibit a diurnal/circadian change in the SCN, with higher levels during the day, although no such changes have been found in human blood samples (Koefoed-Nielsen et al., 2005). In this work we analyzed the pattern of expression of PP2B in the SCN and found it to be relatively stable both under a light/dark schedule or in DD. Therefore, it is unlikely that a change in PP2B protein expression contributed to the time-dependent effect of its inhibition.

The time-dependent effects of calcineurin inhibitors might also be related to changes in pharmacokinetics, which have been described for both CsA and tacrolimus (Baraldo and Furlanut, 2006; Cipolle et al., 1988; Malmary et al., 1992; Ohlman et al., 1993). However, since we have found similar effects for i.p. and i.c.v. administration of different inhibitors, we can conclude that there is direct effect of these compounds on targets in the central nervous system. On the other hand, according to microarray studies, the levels of immunophilins

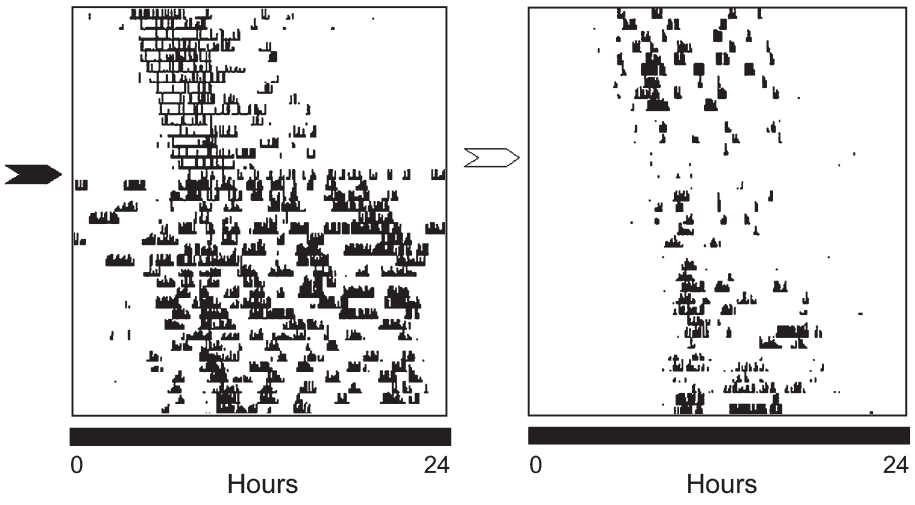

Fig. 4. Chronic inhibition of PP2B activity in the SCN by continuous i.c.v. perfusion in constant darkness. Representative actograms of 1 mM FK506 (left) or vehicle-perfused hamsters (right). Consecutive days are plotted below each other along the *Y* axis. Arrows indicate the day when the osmotic pump was placed in the animal.

(the cytosolic binding proteins for CsA and FK506) that mediate PP2B inhibition appear to be stable throughout the day (Panda et al., 2002).

Taking into account that the feedback loop activity of clock genes in the SCN is regulated by phosphorylation-mediated degradation or translocation of their transcription products, our results suggest that there are components of the molecular clock that are more sensitive to PP2B blockade during the subjective day. Indeed, some clock genes (*per1*, *per2*, *cry1*, *cry2*) peak at this time of day (Reppert and Weaver, 2002) and their activity is modulated by phosphorylation by kinases which include CKIε, whose activity can be increased after dephosphorylation by PP2B (Lowrey et al., 2000; Liu et al., 2002; Eide et al., 2005). Inhibition of PP2B activity in the middle of the resting phase (CT6) should reduce CKIε activity, leading to an earlier *per1,2*/*cry1,2* nuclear accumulation, as it happens in CKIε mutants with reduced circadian period (Lowrey et al., 2000).

In contrast to the results reported here, FK506 induced phase delays of the neuronal firing rate rhythm in mouse SCN slice preparations during the early subjective night (Ding et al., 1998). Since this represents a completely different experimental paradigm, the absence of drug-induced phase delays in our experiments could be explained by a difference in the concentration of drug that reaches the pacemaker cells in each experiment, combined with the known multiple actions of each immunosuppressant drug. Moreover, the absence of specific inputs to the SCN in in vitro slice preparations certainly affects the neurochemical environment in which pharmacological manipulations might change the phase of circadian rhythms; in addition, circadian changes in neuronal firing rhythms do not necessarily correlate with overt locomotor behavior. The relative specificity of immunosuppresant action should also be mentioned. Indeed, since CsA and tacrolimus have been shown to affect multiple signaling events in addition to PP2B, a note of caution should be drawn in that other mechanisms might be responsible for their circadian effects.

Phase shifts induced by glutamate or activators of intracellular Ca^{2+} channels could activate a family of calmodulin-dependent pathways (see Golombek and Ralph, 1995; Golombek et al., 2003), including activation of PP2B. FK506 and CsA might alter the Ca^{2+}-related signal transduction pathway in the SCN, thus affecting the phase of circadian rhythms. Indeed, we found a direct effect of PP2B blockers in the SCN, which induced a decrease in phosphatase activity within 30 min of their administration.

The continuous administration of a drug directly to the SCN via an osmotic pump *in vivo* has been shown to be very useful to assess reversible effects of different compounds on SCN-driven circadian rhythms. In this study, we found a transient disruption of the circadian locomotor activity rhythm during the i.c.v. perfusion of FK506. However, no consistent changes in phase were found after the treatment (and, if found, these changes could be a consequence of the diverse activity patterns during resting hours (Mrosovsky, 1996)). This finding could be interpreted as a direct effect of PP2B inhibition on circadian output, or in the strength of the coupling of clock-related networks responsible for transmitting overt rhythmicity. An alternative hypothetical explanation would be that chronic PP2B inhibition affects the activity of CKIε, an enzyme that has been found to be part of the molecular clockwork in mammals by mediating Per phosphorylation and translocation, and when mutated affects circadian period and phase (Lowrey et al., 2000). However, the clear reduction in amplitude of overt rhythms during chronic PP2B inhibition suggests a change in the strength of molecular cycles, rather than a change in period or phase, resulting in a decreased net output from the clock. Notwithstanding, the results of our chronic infusion may not be related to an effect on circadian clock function and might reflect other unspecific mechanisms of action of chronic FK-506 in the brain. As shown in Fig. 4 and Table 1, animals tend not to recover a completely normal periodicity after the i.c.v. infusion is stopped. Indeed, Qp values from periodogram analysis (which can be interpreted as an index of rhythm robustness) decrease significantly during and after FK-506 infusion. Although the histology did not show any sign of necrosis, this suggests some kind of general alteration in the brain, which can be considered a limitation of the chronic i.c.v. approach. More detailed experiments will be needed in order to determine the precise effect of immunosuppresant infusion on locomotor activity.

It is clear that these experiments do not represent a precise model of the situation for immunosuppressed patients, that usually take two oral doses of the drugs daily. Although we have not tried a similar experiment with CsA because of possible toxic effects in this paradigm, we opted for an i.c.v. administration of FK-506 in order to compare these results to the phase shifting experiments presented before. Indeed, a future experiment should look for the effect of periodic oral administration of immunosuppressants on circadian rhythms, to follow the patients' situation more closely.

In summary, our current data highlight the fact that CsA and FK506 can produce effects on the circadian system, acting on the master circadian clock by inhibiting PP2B activity. The effects include an inhibition of light responsiveness of the clock during the subjective night, as well as a direct alteration of circadian phase when drugs are applied during the subjective day. Consequently, patients under CsA or FK506 treatment might experience not only changes in their endogenous rhythms but also in their ability for the daily synchronization to environmental cues. There is plenty of evidence indicating an improved vascular resistance in PP2B-blocker treated patients that significantly affects the diurnal rhythm of blood pressure (van den Dorpel et al., 1996; Curtis, 2002); however, our results suggest another mechanism for changes in their day/night blood pressure variation as well as for the reported disturbances in their sleep/wake cycle. It is therefore possible that a timely scheduled administration of these drugs could improve patients' quality of life. In addition, the precise role of calcineurin in the molecular signaling pathway of the circadian clock deserves to be fully investigated.

Acknowledgments

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